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Award Number DAMD17-97-1-7265

TITLE: Role of Angiogenic Growth Factor Receptors, Tie 1 and Tie 2, in Metastatic Potential of Breast Cancer

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REPORT DATE: August 1999

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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DTIC QUALITY INSPECTED 4

20001013 074

REPORT DOCUMENTATION PAGEForm Approved
OMB No. 074-0188

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1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE August 1999	3. REPORT TYPE AND DATES COVERED Final (1 Aug 97 - 31 Jul 99)	
4. TITLE AND SUBTITLE Role of Angiogenic Growth Factor Receptors, Tie 1 and Tie 2, in Metastatic Potential of Breast Cancer			5. FUNDING NUMBERS DAMD17-97-1-7265	
6. AUTHOR(S) Lyuba Varticovski, M.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) St. Elizabeth's Medical Center Boston, Massachusetts 02135 E-Mail:			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited				12b. DISTRIBUTION CODE
13. ABSTRACT (Maximum 200 Words) Angiogenesis determines tumor progression and microvessel density correlates with metastatic potential of breast cancer. Normal development of the vascular network in the embryo requires angiogenesis which is regulated by expression of novel endothelial cell receptors, Tie 1 and 2. Tie 2 function is regulated by two ligands, Angiopoietin 1 and its naturally-occurring antagonist, Angiopoietin 2. Expression of these receptors and their ligands has been detected in areas of active vascular remodeling and in wound healing. We generated molecular probes for analysis of expression of Tie 1, Tie2, Angiopoietin 1 and 2 in human and rodent tissues and established cell lines which express the Tie 2 receptor. We also developed a model of human breast cancer in nude rat. We detected expression of Tie receptors in breast tissue and found differences in their expression in malignant and benign breast tumors. These exciting observations suggest that Tie 2 play a role in angiogenesis associated with breast cancer				
14. SUBJECT TERMS Breast Cancer, Angiogenesis, Tie 1 and 2, Angiopoietins				15. NUMBER OF PAGES 54
				16. PRICE CODE
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

FOREWORD

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 8/28/99
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1) Key Research Accomplishments

- The proposed study determined the frequency and pattern of expression of Tie 1 and 2 in primary and metastatic breast cancer which is compared to expression in adjacent tissue and normal breast tissue. We used quantitative RT-PCR on RNA isolated from frozen tissues and immunohistochemistry on paraffin-embedded samples which have been stored at our institution for 5 or more years. We also developed a mouse mammary model in nude rat. Statistical correlation with tumor progression and aggressive behavior of breast cancer is obtained following analysis of clinical data. We have assembled a network of oncologists and pathologists at our institution for this study. Statistical analysis was performed in collaboration with a statistician at TUSM.

2) Reportable Outcomes

- **Tie 1 and Tie 2 expression is induced by injury of endothelial cells (K. Fujikawa and L. Varticovski, manuscript in preparation).** Confluent bovine retinal endothelial cells at passage 3 were injured with a comb which produces a standardized injury to 50% of cells (1). Expression of Tie 1 and 2, b-actin and GAPDH was evaluated by RT-PCR. Tie 1 and Tie 2 expression was induced after EC injury and remained at high levels during EC repair. Therefore, Tie 1 and 2 are induced by direct injury of bovine endothelial cells which may play a significant role in wound healing and small vessel remodeling associated with tumor-associated angiogenesis.

- **Tie expression correlates with reendothelialization following balloon injury of rat carotid artery (2).** The *in vivo* levels of Tie 1 and 2 protein in rat endothelial cells were evaluated in an established model of reendothelialization (3). Normal and balloon-denuded rat carotid arteries (4) were collected at day 3, 7, and 2 and 4 weeks after injury, fixed with methanol and embedded in paraffin. Sections were treated with 3% H₂O₂ to inhibit endogenous peroxidase activity, blocked with normal goat serum, and protein levels were detected by specific rabbit polyclonal antibodies followed by avidin-biotin-immunoperoxidase. Analysis of Tie 1, Tie 2, CD31 and CD34 reveal that normal rat carotid artery reveal high levels of Tie 1 and 2. Expression of Tie 1 and 2 in newly deposited EC recovered at 4 weeks. Therefore, Tie proteins are present in normal rat vascular EC and re-expression of Tie proteins correlates with reendothelialization.

- **Expression of Tie 1 and 2 in normal and malignant breast lesions (5)**, (manuscript in preparation) was examined by RT-PCR and confirmed by Southern blotting using a probe which spans the fibronectin repeat specific for human Tie 1 and 2 (gift of T. Sato, HMS). We developed nested RT-PCR primers based on conserved human and rodent sequences. Increased Tie 1 and Tie 2 were found in breast cancer samples obtained from metastatic disease. Presence of Tie protein in EC of microvessels associated with breast cancer lesions was confirmed using immuno-histochemistry on paraffin-embedded tissues and correlated with microvessel density. We improved the standard methods by treatment of sections with protease which resulted in over 50-fold increase in sensitivity for detection of Tie proteins.

- **Expression of Ang 1 and 2 in breast lesions correlates with malignant phenotype (6).** We determined whether Ang 1 and 2 mRNA can be detected in frozen human breast tissue samples by RT-PCR. We developed specific PCR primers for detection of Ang 1 and Ang 2 in human, rat and mouse tissues. Ang 1 and 2 containing plasmids (G. Yancopoulos, Regeneron) were used as positive controls. The morphology of adjacent tissue for the presence of cancer was examined by histochemistry. Equal amount of RT product was used as determined by expression of GAPDH and β -actin. In addition, we detected Ang 1 and 2 in two estrogen-independent cell lines but not in human estrogen-dependent, MCF7 cells (not shown). Ang 1 expression was detected in all lesions and breast cancer samples contained higher levels of Ang 2 than Ang 1. These data suggest that the level of expression of the pattern of Ang 1 and 2 expression may provide a marker of aggressive behavior of breast cancer.

- **Angiopoietin-1 is a survival Factor for human endothelial cells (7).** Ang-1 binding to its receptor, Tie2, is not mitogenic and both receptor and ligand are expressed in quiescent tissues. Activation of Tie2 receptor leads to autophosphorylation, interaction with several adaptor molecules and activation of PI 3-kinase but no detectable mitogenic response. We showed that Angiopoietin-1 is a survival factor for endothelial cells and induces PI 3-kinase-dependent activation of Akt. Apoptosis of growth factor deprived endothelial cells was PI 3-kinase dependent and inhibited by Angiopoietin-1. Ceramide, an intermediate of the apoptotic pathway, interferes with Akt activation by unknown mechanism. Ceramide also induced endothelial cell death and abolished Angiopoietin-1-mediated activation of Akt and cell survival. In addition, Angiopoietin-1-induced migration of endothelial cells was blocked by PI 3-kinase inhibitors, whereas activation of MAPK/ERKs was independent of PI 3-kinase activity. These findings indicate that PI 3-kinase lipid products are key mediators of the biological effects of Angiopoietin-1 on endothelial cell survival and migration.

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Role of PI 3-kinase in Angiopoietin-1-Mediated Migration and Attachment-Dependent Survival of Endothelial Cells.

Running title: Tie2 biological effects require PI 3-kinase activation.

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ABSTRACT

Angiopoietin-1 is a unique growth factor which induces Tie2 receptor autophosphorylation and interaction with signal transduction molecules, GRB2 and p85 subunit of PI 3-kinase, but no detectable mitogenic response. Here we show that PI 3-kinase-dependent activation of Akt and attachment to extracellular matrix are required for angiopoietin-1 mediated endothelial cell survival. Apoptosis of growth factor-deprived cells grown in monolayer was decreased by angiopoietin-1 and correlated with Akt activation. In contrast, angiopoietin-1, bFGF or VEGF failed to protect cells in suspension culture. Ceramide, an intermediate of several apoptotic pathways, interferes with growth factor-mediated Akt activation. Ceramide induced endothelial cell death and abolished angiopoietin-1-mediated activation of Akt and the effect on cell survival. In addition, we found that PI 3-kinase activity is necessary for migration of endothelial cells in response to angiopoietin. A transient activation of MAPK/ERKs was also detected within 10 minutes after stimulation with angiopoietin-1. In contrast to VEGF-mediated biological effects, inhibition of MAPK/ERKs by PD98059 in endothelial cells did not affect angiopoietin-1 mediated survival or migration. These findings indicate significant differences in intracellular signaling between VEGF and angiopoietin-1 and that PI 3-kinase lipid products are key mediators of the biological effects of angiopoietin-1.

INTRODUCTION

Sequential expression of endothelial growth factors and receptors of the tyrosine kinase superfamily determines development of the vascular system. Angiogenesis in the embryo is regulated by a novel family of receptor tyrosine kinases, *tie* [1, 2]. The *tie* family of receptors consists of two members, Tie1 and Tie2 (Tek), which are predominantly expressed in endothelial cells [3-6]. Functional disruption of Tie1 causes perinatal lethality with severe defects in vascular integrity and structure, whereas lack of Tie2 is lethal in early embryonic development with defects in microvascular development [7-9]. mRNAs for receptors are expressed at lower levels postnatally; however, their role in normal adult vascular endothelium is not well understood. The ligands for Tie1 receptor tyrosine kinase have not been identified. The two naturally occurring ligands for Tie2, Angiopoietin-1 (ang1) and Angiopoietin-2 (ang2), have biologically opposing effects. Ang1 induces Tie2 tyrosine kinase activity and this effect is blocked by Ang2 [10, 11]. Ang1 deletion or Ang2 overexpression result in embryonic lethality with defects similar to those observed due to disruption of Tie2 [11, 12].

Receptor tyrosine kinases induce diverse biological activities including migration, differentiation and proliferation. Recent studies indicate that the mitogenic response is frequently accompanied by activation of signal transduction pathways which block apoptosis. Activation of PI 3-kinase by receptor and oncogene protein-tyrosine kinases was initially linked to mitogenesis. Subsequent studies showed that lipid products of PI 3-kinase also participate in regulation of cell survival by activation of serine/threonine kinase, Akt [13, 14].

Although an initial report suggested that activated Tie2 does not associate with PI 3-kinase [15], subsequent studies [16] and our data presented here confirm that activation of Tie2 leads to PI 3-kinase-dependent activation of Akt. Angiopoietin-1 prevented apoptosis of human umbilical vein and microvascular endothelial cells in monolayer and this protection was sensitive to PI 3-kinase inhibitors, Wortmannin and LY294002, at concentrations which are specific for mammalian Type I PI 3-kinase. PI 3-kinase activity was also necessary for Angiopoietin-1-induced migration of endothelial cells. These studies indicate a critical role for PI 3-kinase lipid products in signal transduction by Tie2

and support the hypothesis that interaction of Angiopoietin-1 with Tie2 in adult vascular system is essential for endothelial cell survival, migration and vascular repair.

MATERIALS AND METHODS

Reagents. Purified recombinant Angiopoietin-1* and soluble Tie2 receptor (sTie2R), which is a fusion protein of extracellular domain of human Tie2 and Fc of human immunoglobulin, were kindly supplied by G. D. Yancopoulos (Regeneron, NY). Angiopoietin-1* used in all experiments was modified from the original Angiopoietin-1 [11] and was designated as ang1 in this study. Human recombinant vascular endothelial cell growth factor (VEGF)₁₆₅ was from Genentech, Inc. (San Diego, CA). Wortmannin (WM) was from Sigma Chemicals (St. Louis, MO). LY294002 (LY), C2-ceramide (C2-C), and dihydro C2-ceramide (DhC2-C) were from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA). PD98059 (PD) was from Calbiochem-Novabiochem Corporation (San Diego, CA). Fluorescein isothiocyanate (FITC)-conjugated annexin V and propidium iodide (PI) were from Oncogene Research Products (Cambridge, MA). Rabbit polyclonal anti-human Tie1 and Tie2, and goat polyclonal anti-pan-Akt antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-tyrosine (4G10) and pan-ERK antibodies were from Upstate Biotechnology (Lake Placid, NY). Rabbit polyclonal anti-phospho-Akt (pAkt) and anti-phospho-ERK (pERK) were from New England Biolabs (Beverly, MA) and from Promega (Madison, WI), respectively.

Cell culture. Human umbilical vein endothelial cells (HUVEC) isolated from human umbilical cords [17] were grown in medium 199 (M199) (Bio Whittaker, Walkersville, MD) containing 20% fetal bovine serum (FBS) and bovine brain extract with heparin (Clonetics Corp., San Diego, CA) as previously reported [18]. HUVEC were used within passages three to six. EA.hy 926, a HUVEC and A549 human lung carcinoma hybrid cell line, was a gift from Dr. C-J S. Edgell [19]. EA.hy 926 cells were maintained in Dulbecco's modified Eagle's medium (DME) supplemented with 10% FBS. Human microvascular endothelial cells (HMEC) were purchased from American Type Culture Collection (ATCC) (Rockville, MD), and grown in minimal essential media supplemented with 10%

FBS. For some experiments, FBS was dialyzed overnight against PBS (D-FBS) using 30 kDa cut-off membrane (Amersham Pharmacia Biotech, Inc, Piscataway, NJ).

Detection of cell viability. $1-2 \times 10^4$ of HUVEC or HMEC were seeded per well in complete media into fibronectin-like engineered protein polymer (FNP) coated 96-well plates (Sigma) or 1% gelatin coated tissue culture plates (Fisher Scientific, New York, NY). After 24 hours, cells were washed twice with PBS and placed in media containing 0.5% D-FBS and 1% bovine serum albumin (BSA) with/without growth factors for indicated time. For Ang1 competition studies, one hundred-fold excess of soluble Tie2 receptor (sTie2R) was preincubated with Ang1. For ceramide-induced apoptosis, HUVEC or EA.hy 926 cells were seeded in complete culture media into FNP coated or Primaria 96-well plates (Fisher), respectively. Cells were washed and placed in 2% FBS or 1% BSA, respectively, for indicated time with C2-ceramide or dihydro C2-ceramide 30 minutes prior to treatment with growth factors. The final concentration of ethanol in all experimental conditions and control media was adjusted at 0.2%. Cell viability was determined using CellTiter 96 AQ non-radioactive assay (MTS assay) kit (Promega). MTS (3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)2H-tetrazolium)/phenazine methosulfate mixture was added to cells and incubated for 2-4 hours at 37°C before measuring absorbance at 490 nm. Sextuplet wells were used for each experimental condition.

For viability of cells in suspension, HUVEC or HMEC grown to 90-100% confluency were detached with PBS/0.5% EDTA, pH 7.6. Cells were washed 2 times in media with 1% BSA and plated at 1×10^5 cells/ml in non-tissue culture treated 96-well plates in 1% BSA with or without ang1 at 200 ng/ml, VEGF at 30 ng/ml, bFGF at 3 ng/ml or 10% FBS for 20-24 hours. MTS assay was performed as above. Quadruplicate or sextuplet wells were used for each experimental condition.

Measurement of DNA synthesis. [^3H] thymidine incorporation was tested in growth factor deprived endothelial cells cultured in FNP-coated 96-well plates following addition of 5 $\mu\text{Ci/ml}$ of [^3H] thymidine to cells for 6 hours. After washing twice with PBS, samples were precipitated in 10% trichloroacetic acid, the sediment was lysed in 0.1N sodium

hydroxide, and radioactivity counted. Sextuplet wells were used for each experimental condition.

Detection of DNA fragmentation. DNA fragmentation was analyzed by colorimetric assay using diphenylamine [20]. HUVEC were cultured to 70% confluency on gelatin coated dishes and media was replaced for 24 hours to 1%BSA with or without growth factors or 10%FBS. Detached floating and adhered cells were lysed in the lysis buffer (10 mM Tris-HCl, pH 7.5, 10 mM EDTA, 0.5% Triton X-100) on ice, and centrifuged at 12,000 X g for 20 minutes. Supernatants and pellets were collected separately. The fragmented and genomic DNA from supernatants and resolubilized pellets, respectively, were precipitated overnight in 3% perchloric acid at 4°C. Following centrifugation, DNA pellets were suspended in 50 µl of 6% perchloric acid, and incubated at 70°C for 20 minutes. One hundred microliters of diphenylamine solution (1.5% (w/v) diphenylamine, 1.5% sulfuric acid and 0.01% acetaldehyde in acetic acid) was added and samples were incubated overnight in the dark. Absorption at 600 nm was measured and DNA fragmentation was calculated as percent of total DNA [(OD supernatants/OD supernatants + OD pellets) X 100].

Annexin V binding. Subconfluent HUVEC cultured on gelatin-coated tissue culture plates were washed twice with PBS and cultured in media with 0.5% D-FBS and 1% BSA with/without growth factors. After 24 hours, detached floating cells were collected by centrifugation, and adhered cells were trypsinized and harvested. Cells were combined, suspended in M199 containing 5% FBS and incubated with annexin V-FITC for 15 minutes at room temperature. PI was added on ice as per manufacturer's instructions. Percentage of apoptotic cells was determined as percent of annexin V positive cells using FACScan (Becton Dickinson, Franklin Lakes, NY).

Endothelial cell migration assay. A 48-well microchemotaxis modified Boyden chamber (Neuroprobe, Inc, Cabin John, MD) was used for migration assays as described [21]. HUVEC cultured to confluency were trypsinized and suspended in M199 containing 1% FBS at $2.5-5 \times 10^6$ /ml. 50 µl of cell suspension was added to the upper chamber above the

polyvinylpyrrolidone-free polycarbonate filters which had a pore size of 8 μm (Laboratory & specialty products group, Livermore, CA) coated with 0.2% gelatin in PBS. 25 μl of test substances were added to the bottom chamber. After 4-5 hours at 37°C, the filters were removed, and nonmigrating cells were scraped. Cells which migrated were fixed in 100% methanol and stained with Giemsa (Sigma). Filters were mounted on glass microscope slides and cells which migrated were counted in three random high power fields in each well. Duplicate to quadruplicate studies were performed for each experimental condition.

Western blot analysis. HUVEC were seeded at 70% confluency in complete media for 24 hours. Cells were washed twice in PBS, placed in M199 with 1% BSA or 0.5% D-FBS/1% BSA for additional 16-20 hours and stimulated with growth factors for indicated times. The inhibitors were added to cells 30 minutes prior to growth factor stimulation. Cells were lysed in HEPES, pH 7.4, containing 1% NP-40 and protease inhibitors [22]. Protein concentration was determined by Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA) using bovine IgG as a standard. 50 μg of whole cell lysate (WCL) protein was fractionated by 12% SDS-PAGE and electrophoretically transferred onto nitrocellulose membrane. Membranes were blocked for 1 hour at room temperature (7-8% non-fat milk and 2% BSA in PBS, 0.2% Tween-20 (PBS-T)) and incubated with anti-phospho-ERK (1:10,000), anti-pan-ERK (1:5,000), anti-phospho-Akt (1:2,500) at 4°C overnight, or anti-pan-Akt antibodies (1:2,000) for 2 hours at room temperature. Membranes were washed 4-5 times for 10 minutes with PBS-T and reacted with horse radish peroxidase-conjugated second antibodies: anti-rabbit (Signet Labs, Dedham, MA and Santa Cruz), anti-goat (Santa Cruz) or anti-mouse IgG (Sigma), as needed, for 40 minutes. Immunoreactive bands were detected using ECL reagent (Pierce, Rockford, IL) as per manufacturers' instructions.

For analysis of phosphorylated Tie2, 300 μg of WCL protein was incubated with anti-phospho-tyrosine antibodies at 4°C for 2 hours, and immune complexes collected on Protein A/Sepharose beads, washed sequentially with PBS, lysis buffer, and twice with PBS, resolved on SDS gel, transferred onto nitrocellulose membranes, and probed with anti-Tie2 (1:1,000) antibodies [23]. Immunoreactive bands were detected as above.

PI 3-kinase activity. PI 3-kinase activity was determined in anti-phospho-tyrosine immunoprecipitates from 500 µg of WCL protein as described [22]. PI 3-kinase reaction was performed at 37°C for 20 minutes using micellar suspension of PI, PIP₂ and PS (1:1:1) as substrates/carrier with 150 µM ATP and 20 µCi [γ -³²P]-ATP/sample. Phospholipids were extracted into chloroform, dried under nitrogen, separated by TLC in n-Propanol: Acetic acid: H₂O (64:1:34) and PIP₃ product was identified by autoradiography and quantified by counting TLC spots eluted in scintillation fluid.

RESULTS

Ang1 is a survival factor for growth factor deprived human endothelial cells. VEGF and bFGF are mitogenic for vascular endothelial cells and rescue growth factor-deprived endothelial cells from apoptosis [24, 25]. To determine whether ang1 is a survival factor for endothelial cells, cell viability of growth factor-deprived and control endothelial cells treated with ang1 or VEGF was estimated using MTS assay which reflects mitochondrial integrity [26]. Under phase contrast microscopy, HUVEC maintained in 0.5% D-FBS/1% BSA for 24-48 hours were rounded and shrunken, had membrane blebbing, and detached from the plates as previously described [24]. As seen in Figure 1A, viability of HUVEC maintained for 24 hours in low serum was increased by ang1. The maximal effect was achieved with 100 ng/ml of ang1. The survival effect of ang1 was blocked by preincubation with recombinant soluble Tie2 receptor extracellular domain (sTie2R) which competes with binding to Tie2. The recovery of cells grown in 10% FBS (5.2×10^4 /well) and in low serum (2.63×10^4 /well) correlated with the results obtained using MTS assay. These data indicate that ang1 specifically increases the viability of HUVEC by activation of Tie2 receptor. HMEC were more sensitive to the effects of ang1 (Fig. 1B). Consistent with previous reports [24, 27], cytoprotection of growth factor-deprived HUVEC and HMEC was also induced by VEGF (Fig. 1A and B). Ang1 had approximately 50% of the effect induced by VEGF in both cell types. Combination of ang1 and VEGF was not synergistic and had an additive effect (data not shown).

To confirm whether ang1 increases survival of endothelial cells by interfering with apoptosis, we performed quantitative analysis of DNA fragmentation and flow cytometric analysis for annexin V-FITC binding of cells treated with ang1. Growth factor-deprived HUVEC had 30.3% DNA fragmentation, Ang1 at 500 ng/ml reduced DNA fragmentation to 22.3% which is statistically significant under the conditions of our assay whereas FBS further reduced DNA damage to 19% (Fig. 2A). Similar results were obtained using 100 ng/ml of ang1 (data not shown). Annexin V binds to phosphatidyl serine which normally is maintained on the inner leaflet of the plasma membrane but appears on the outer leaflet at early stages of apoptosis [28]. Time-dependent increase in annexin V positive endothelial cells maintained in low serum was significantly reduced

by ang1 (23% to 13%), which was almost as effective as was 10% FBS (10%) (Fig. 2B). These results indicate that ang1 has a survival effect on growth factor deprived primary human endothelial cells.

No mitogenic effect of ang1 on endothelial cells, as measured by [³H] thymidine incorporation, was detected (data not shown). The lack of ang1 mediated mitogenic response has been previously documented [16, 21].

Ang1 induced survival of endothelial cells requires PI 3-kinase activity. Activation of PI 3-kinase leads to initiation of Akt-dependent survival pathway [29]. To determine whether PI 3-kinase mediates anti-apoptotic signal of ang1, we used LY294002, which inhibits Type I PI 3-kinase with IC₅₀ of 1-5 μ M [30]. LY294002 accelerated the death of growth factor-deprived endothelial cells in a dose (Fig. 3A) and time (Fig. 3B) dependent manner. Twenty percent reduction in cell viability was detected in 20 hours with 5 μ M LY294002, whereas 20 μ M resulted in 40% increase in cell death (Fig. 3A and B). As seen in Fig. 3C, 20 μ M LY294002 completely blocked the antiapoptotic effect of ang1 and most of the effect of VEGF. Contrary to previously reported requirement of PI 3-kinase activity for VEGF-mediated cell survival [24], we observed only partial inhibition by LY294002 which could be due to VEGF-induced mitogenic response under the conditions of our assay. These data indicate that activation of PI 3-kinase is required for ang1-mediated cytoprotection of endothelial cells. The lack of ang1 mediated survival effect on LY294002-treated cells was confirmed by analysis of DNA fragmentation (Fig. 3D).

Ang1 induces PI 3-kinase-dependent activation of Akt in endothelial cells. The serine/threonine kinase Akt mediates PI 3-kinase dependent activation of the anti-apoptotic pathway. Low levels of phospho-Akt (p-Akt) were detected in quiescent cells. Although the antibody used for detection of p-Akt (Ser473) does not distinguish the phosphorylation on Thr308 that is required for full activation of Akt *in vivo*, the observed increase in p-Akt content correlates with activation of Akt serine/threonine kinase activity [31]. Activation of Akt was maximal in 10 minutes and persisted for 6 hours. Higher levels of Akt activation were observed in response to FBS (Fig. 4A, upper panel). Expression of Akt did not change during the experiment and constant amount of

protein was loaded in each lane as demonstrated by probing the same membrane with pan-Akt antibodies (Fig. 4A, lower panel). Ang1 also induced a modest 1.3-fold increase in anti-phospho-tyrosine associated PI 3-kinase activity in 10 minutes which declined to baseline level after one hour. Activation of Akt and PI 3-kinase was abrogated by 20 μ M LY429002 or 30 nM Wortmannin (Fig. 4B and data not shown). We conclude that PI 3-kinase-dependent activation of Akt is required for ang1-mediated cytoprotection of human endothelial cells.

Ceramide induces apoptosis of endothelial cells which is not reversed by ang1.

Intracellular levels of ceramide rise in response to apoptosis induced by TNF α , Fas ligand, or UV irradiation [32]. We tested the effect of cell-permeable C2-ceramide and its inactive analogue, C2-dihydroceramide, on survival of endothelial cells. Both were added to HUVEC or EA.hy 926 cells in media containing 2% FBS or 1% BSA. C2-ceramide induced a time-dependent loss of viability as compared to control cells (Fig. 5A, B). C2-ceramide induced loss of cell viability of HUVEC with IC₅₀ of 50 μ M. Similar observations were extended to EA.hy 926 cells, although these cells were more resistant to the effects of C2-ceramide; the IC₅₀ was 100 μ M (Fig. 5B). Dihydro C2-ceramide at 100 μ M had no significant toxicity in either cell type. Ang1 failed to prevent apoptosis of cells treated with C2-ceramide whereas 10% FBS restored cell viability even in presence of ceramide (Fig. 5C).

Ceramide has multiple intracellular targets and could participate in apoptotic pathway at many levels. Therefore, we tested whether ceramide has an effect on activation of Tie2, as assessed by ang1-induced autophosphorylation of Tie2. Autophosphorylation of Tie2 was evident in quiescent HUVEC in spite of 24 hours of growth factor deprivation and correlated with low levels of activated Akt (compare Fig. 5 Panels D and E). Increased Tie2 autophosphorylation was observed in ang1 but not FBS-stimulated HUVEC and was not altered by C2-ceramide (Fig. 5 D). Therefore, ceramide does not decrease ang1-induced Tie2 autophosphorylation.

C2-ceramide treated endothelial cells had no detectable levels of phospho-Akt and ang1 failed to induce Akt activation in these cells (Fig. 5E). Interestingly, although ceramide blocked FBS-mediated activation of Akt, it had no effect on FBS-induced cell

survival. Ceramide has been described to interfere with activation of Akt without affecting PI 3-kinase activity in other cell types [33]. Therefore, ceramide inhibits Akt activation in endothelial cells without interfering with activation of Tie2 which supports our conclusion from previous experiments that PI 3-kinase/Akt pathway is required specifically for ang1-mediated endothelial cell survival.

Endothelial cell growth factors fail to protect cells from apoptosis in suspension. VEGF was reported to mediate cytoprotection in an anchorage-dependent manner [27]. We tested whether ang1, VEGF, bFGF or FBS confer resistance to apoptosis of cells in suspension. HUVECs were collected by 0.5% EDTA/PBS, treated with growth factors or 10% FBS and plated in flat bottom untreated 96-well plates. Attachment of FBS-treated cells was evident within 4-5 hours, which indicates that FBS contains components of extracellular matrix which promote cell adhesion. In contrast, control and growth factor-treated cells were clumped and had morphological features of anoikis as described previously (ibid). Ang1, bFGF and VEGF did not protect HUVEC from anoikis whereas addition of 10% FBS resulted in nearly 10-fold increase in viability detected by MTS (data not shown). Similar results were obtained using human microvascular cells. These data suggest that matrix attachment is required for ang1 and other growth factor-induced protection from apoptosis.

Ang1 induces transient activation of MAPK/ERKs which is independent of PI 3-kinase and does not correlate with biological effects of ang1. In many cells, including endothelial cell, activation of PI 3-kinase leads to activation of MAPK/ERKs [34, 35] and PI 3-kinase inhibitors block VEGF-mediated activation of MAPK/ERKs [36]. In contrast to a previous report which suggested that ang1 does not activate MAPK/ERKs [16], phosphorylation of ERK 1 and 2 was detected within 10 minutes in serum starved endothelial cells after addition of ang1 (Fig. 6A). This response was transient and returned to baseline within 1 hour. Ang1 induced activation of MAPK/ERKs was independent of PI 3-kinase activity because it was not changed by LY294002 (Fig. 6B) but blocked by MEK inhibitor, PD98059 at 10 μ M (data not shown). As previously reported, endothelial cell viability was decreased by $\pm 30\%$ in 10 μ M PD98059 [36]. However, ang1-

mediated protection from apoptosis (Fig. 6C) or migration (data not shown) were not affected. Therefore, in contrast to VEGF-mediated signal transduction [36], activation of MAPK/ERKs is not required for ang1-mediated cell survival or migration.

PI 3-kinase activity is also necessary for ang1-induced migration of endothelial cells. A recent study showed that ang1 is chemotactic for endothelial cells, an effect which is specifically blocked by Ang2 [21]. Activation of PI 3-kinase has been implicated in migration and its lipid products induce migration of macrophages [37]. We tested whether PI 3-kinase lipid products participate in ang1-induced chemotactic response of endothelial cells. Ang1 induced 55-60% increase in migration which was almost as efficient as FBS which induced a 70% increase and PI 3-kinase inhibitors (10 μ M LY294002 or 30 nM Wortmannin) blocked ang1-induced migration (data not shown). Endothelial cell viability over the short duration of this assay was not changed by the inhibitors. These data indicate that PI 3-kinase lipid products participate in ang1 mediated chemotactic response of endothelial cells.

DISCUSSION

Accumulation of Type I PI 3-kinase lipid products in response to growth factors and cytokines is necessary for recruitment of Akt to the plasma membrane [38] where it is fully activated following phosphorylation by PDK1 and PDK2 serine/threonine kinases [13, 29, 39]. Active Akt blocks the apoptotic cascade at several points. Phosphorylation of BAD by Akt and subsequent association with 14-3-3 remove the protein from Bcl-2 allowing its effect on cell survival [14]. In addition, Akt phosphorylates and inactivates Caspase 9 and a member of the Forkhead family of transcription factors, FKHRL1, further arresting progression of the cell death cascade [40, 41]. We confirmed previously reported activation of Akt by ang1 [16] and demonstrated that activation of PI 3-kinase is required for ang1 mediated activation of Akt and endothelial cell survival. The anti-apoptotic effect of ang1 and activation of Akt were blocked by PI 3-kinase inhibitors, Wortmannin and LY294002, at concentrations which specifically block Type I PI 3-kinase activity [30]. These data indicate that PI 3-kinase-dependent activation of Akt mediates the anti-apoptotic signal of ang1.

Ceramide is a secondary sphingomyelin metabolite which is generated in apoptosis induced by stress, IL-1, TNF α , and FAS ligand [32]. Although there is controversy regarding the exact mechanism of its actions, ceramide has multiple intracellular targets and blocks activation of Akt [33]. Cell permeable C2-ceramide induced apoptosis and blocked ang1-mediated activation of Akt and survival of endothelial cells. Ceramide also downregulates expression of cell surface receptor for Fas Ligand [42]. However, ceramide had no effect on ang1 mediated autophosphorylation of Tie2. These data provide further evidence that ang1-mediated activation of Akt is required for survival of endothelial cells.

We observed that Tie2 autophosphorylation in growth factor-deprived endothelial cells correlated with low levels of p-Akt which was abrogated by PI 3-kinase inhibitors. Tie2 phosphorylation in tissues which maintain quiescent endothelium has been previously reported [43, 44] while autophosphorylation of VEGF receptors is not detected in unstimulated endothelial cells [18]. In addition, LY294002 induced apoptosis of quiescent endothelial cells. These data suggest that constitutive activation of PI 3-kinase

may be required for viability of quiescent endothelial cells, and that availability of ang1 [44] may account for active Akt in quiescent cells. Because ang1 does not induce a mitogenic response, constitutive Tie2 activation may contribute to maintenance and repair of adult vascular endothelium. Negative regulation of Tie2 signaling in selective areas may be provided by PKC-mediated release of the extracellular domain of Tie2 [45] and local release of Ang2 which compete for ligand binding [2, 11].

Consistent with previous reports, endothelial cells in suspension underwent anoikis, a form of programmed cell death of adherent cells in the absence of attachment. Constitutively active Akt but not VEGF reduced death of endothelial cells in suspension [24, 27]. Mice which lack ang1 or its receptor have impaired endothelial cell-matrix interactions [7-9, 12] which suggest that Tie2 signal transduction participates in this process. Therefore, we tested whether ang1 could rescue endothelial cells from apoptosis in the absence of attachment. We found that ang1 as well as bFGF or VEGF were unable to rescue endothelial cells from anoikis. These findings suggest a general principle that growth factor-mediated activation of Akt is required for survival of endothelial cells and that interaction with extracellular matrix may have independent effect on cell survival.

Activation of PI 3-kinase is also linked to cytoskeletal rearrangements and chemotaxis [46-48]. Mutations of the PDGF receptor which abolish p85 binding, impair PDGF-dependent chemotaxis [49, 50], and addition of phospholipid products of PI 3-kinase to intact cells induces macrophage migration [48]. The mechanism of PI 3-kinase mediated cell motility is not well characterized, but may involve activation of PKC ζ and small GTP binding protein, Rac [48, 51, 52]. We found that ang1-mediated activation of PI 3-kinase is necessary for the chemotactic effect of ang1. The mechanism of PI 3-kinase mediated cell motility in response to ang1 is not known and requires further study.

Ang1 also induced significant but transient activation of MAPK/ERKs which was abolished by MEK inhibitor, PD98059 but was resistant to PI 3-kinase inhibitors. In addition, PD98059 had no effect on ang1-mediated cytoprotection or migration. These data indicate that, in contrast to VEGF-mediated signal transduction [36], activation of MAPK/ERK is PI 3-kinase independent. Ang1 mediated autophosphorylation of Tie2 is reported to induce interaction with several adaptor proteins, including Grb2 [15, 16],

which may recruit the SOS/p21^{ras} pathway and subsequently activate MAPK/ERKs (Fig. 7).

Initial enthusiasm regarding a role for ang1 in embryonic development of the vascular system was tempered by observations that ang1 is not mitogenic for cells which express Tie2 receptor. However, subsequent studies, including ours, indicate that activation of Tie2 receptor plays a critical role in endothelial cell survival and migration. Ang1 is the first known growth factor which induces activation of PI 3-kinase without leading to a mitogenic response. Studies in mice which lack PI 3-kinase regulatory subunit, p85 α , also suggest that cell survival and entrance into the cell cycle may be regulated separately by 3' polyphosphoinositides [53]. Therefore, activation of Tie2 provides a unique model for understanding intracellular signaling in which PI 3-kinase-dependent cell survival and migration can be separated from a mitogenic response.

ACKNOWLEDGMENTS

We are grateful to Dr. Yoko Inai for help with DNA fragmentation assay, Neil Tritman for isolation of endothelial cells, Donna-Marie Mironchuk for making the figures and Dr. Irwin M. Arias for critical review of the manuscript.

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FIGURE LEGENDS

Figure 1

Ang1 restores viability of growth factor deprived primary human endothelial cells.

Panel A: HUVEC; **Panel B:** HMEC. Cell viability was estimated by MTS assay 24 hours after induction to quiescence. The results shown are one of four independent experiments for HUVEC and three independent experiments for HMEC. Data are expressed as relative to control cells grown in 10% FBS (for HUVEC) or 100 ng/ml VEGF (for HMEC) and represent means \pm S.D. from sextuplet wells. Survival of HMEC in 10% FBS was 20-30% higher than in VEGF.

Figure 2

Ang1 is a survival factor for growth factor deprived endothelial cells.

Panel A: DNA fragmentation quantitated by colorimetric assay. HUVEC were grown to 80% confluency and placed in 10 % FBS or 0.5% D-FBS with 1% BSA and ang1 (500 ng/ml) or VEGF (100 ng/ml) for 24 h. Percentage of fragmented DNA as compared to cells maintained in 10% FBS is presented as means \pm S.E. from four independent experiments.

Panel B: Ang1 reduces the number of annexin V positive cells. HUVEC treated with ang1 (500 ng/ml) in 0.5% D-FBS with 1% BSA or 10% FBS in M199 media for 24 h were treated with FITC-conjugated annexin V and PI and analyzed for fluorescence intensity by FACScan. Data represent means \pm S.E. of annexin V positive cells from three independent experiments.

Figure 3

Ang1 induced activation of PI 3-kinase is required for endothelial cell survival.

Panel A: Effect of PI 3-kinase inhibitor, LY294002, on viability of HUVEC. Cells were incubated with indicated concentrations of LY294002 for 20 h. Cell viability was determined by MTS assay as described under legends to Figure 1. Data are presented as percent of full viability as means \pm S.D. from sextuplet wells.

Panel B: Time-dependent effect of LY294002 on viability of HUVEC. Cells were deprived from serum and treated with 5 (triangles) or 20 (circles) μ M of LY294002 for indicated times. Cell viability was measured by MTS assay as described above. Data are expressed as relative to vehicle treated controls and represent means \pm S.D. from sextuplet wells.

Panel C: Endothelial cell survival in response to ang1 requires active PI 3-kinase.

HUVEC were treated with 20 μ M of LY294002 or 0.1% DMSO for 30 min prior to addition of ang1 (500 ng/ml), VEGF (100 ng/ml), or 10% FBS. After 24 h, cell viability was assessed by MTS assay. Data are expressed relative to cells grown in 10% FBS as means \pm S.D. from three independent experiments. Statistical analysis was performed using student t test; NS = not statistically significant.

Panel D: Ang1 induced protection from DNA fragmentation requires PI 3-kinase activity. HUVEC cultured in 60-mm dishes were placed in media containing 0.5% D-FBS/1% BSA for 24 h, treated with 20 μ M LY294002 for 30 min and subsequently with ang1 (500ng/ml), VEGF (100 ng/ml) or 10% FBS for additional 24 h. Percentage of fragmented DNA is presented as means \pm S.E. from three independent experiments.

Figure 4

Ang1 induces PI 3-kinase dependent activation of Akt.

Panel A: HUVEC were induced to quiescence for 24 hours and stimulated with ang1 (100 ng/ml) for 0 to 6 h (lanes 2-5), or 10% FBS for 10 min (lane 1) and lysed as described under Materials and Methods. 50 µg of protein was fractionated by SDS-PAGE, transferred onto nitrocellulose membrane and probed with anti-p-Akt antibodies (upper panel). The membrane was neutralized with 15% H₂O₂ for 20 minutes and reprobed with anti-pan-Akt antibodies to ensure the equal protein loading and expression of Akt in all lanes (lower panel).

Panel B: Quiescent HUVEC were pretreated with 20 µM of LY294002 for 30 min prior to stimulation with ang1 or 10% FBS for 10 min. Cell lysates were analyzed with anti-p-Akt antibodies as above.

Figure 5

Ceramide induces apoptosis of endothelial cells which is not reversed by ang1.

Panels A and B: Effect of C2-ceramide on viability of endothelial cells. HUVEC (**Panel A**) and EA.hy 926 cells (**Panel B**) were treated with 10 (closed circles), 50 (open circles) or 100 (closed triangles) μ M C2-ceramide, or 100 μ M C2-dihydroceramide (open triangles) for indicated times. Cell viability was assessed by MTS assay. The results shown are one of two independent experiments. Data are expressed as % viability relative to vehicle treated controls and represent means \pm S.D. from sextuplet wells.

Panel C: Ang1-induced cell survival of endothelial cells is blocked by C2-ceramide. HUVEC were pretreated with 100 μ M C2-ceramide or C2-dihydroceramide for 30 min and grown in the presence of ang1 or 10% FBS for additional 16 h. Cell viability was measured by MTS assay and data expressed relative control cells grown in 10% FBS. The results shown are means \pm S.E. from three independent experiments using 6 wells for each experimental condition.

Panel D: C2-ceramide does not interfere with ang1-induced autophosphorylation of Tie2. Quiescent HUVEC (lanes 1 and 2) or cells pretreated with 100 μ M of C2-ceramide for 30 min (lanes 3 and 4), were stimulated with ang1 (lanes 2 and 4) or 10% FBS as a control (lane 5) for 10 min. Anti-phospho-tyrosine immune precipitates were obtained from 300 μ g of whole cell lysate protein and western blot was performed using anti-Tie2 antibodies.

Panel E: C2 ceramide blocks ang1-induced Akt activation. Western blot on 30 μ g of protein from whole cell lysate obtained from C2-ceramide and ang1 or FBS treated HUVEC were probed with anti-phospho-Akt antibodies.

Figure 6

Ang1 induces activation of MAPK/ERKs which is independent of PI 3-kinase.

Panel A: Ang1 induces a transient MAPK/ERKs activation. Quiescent HUVEC were stimulated with ang1 for 0 to 6 h and analyzed by Western blotting using phospho-specific anti-ERK antibodies.

Panel B: Ang1-induced MAPK/ERKs activation is independent of PI 3-kinase. The same blot in Figure 4B was reprobed with anti-phospho-ERK antibodies (upper panel). The membrane was stripped with 10X Ponceau reagent for 15 min following by 1 N NaOH solution for 5 min, washed extensively and reprobed with anti-pan-ERK antibodies (lower panel).

Panel C: Ang1 effect as a survival factor is independent of MAPK/ERK activation. Cells were grown for 24 hours in 10% FBS, 1% BSA or 1% BSA with 100 ng/ml ang1 for 20 hours with or without 1 or 10 μ M PD98059. Cell viability was estimated by MTS assay and compared to cells grown in 10% FBS.

Figure 7

Ang1-mediated activation of signal transduction pathways in endothelial cells.

Activation of Tie2 receptor leads to activation of MAPK/ERK and PI 3-kinase by functionally independent pathways. Akt is a downstream effector of PI 3-kinase, and its inhibition blocks ang1-induced cell survival. Ang1 induces migration of endothelial cells which is also dependent on PI 3-kinase activity. Neither cell survival nor migration require activation of MAPK/ERKs. Thus, PI 3-kinase regulates biological effects of ang1 in endothelial cells.

Fig. 1

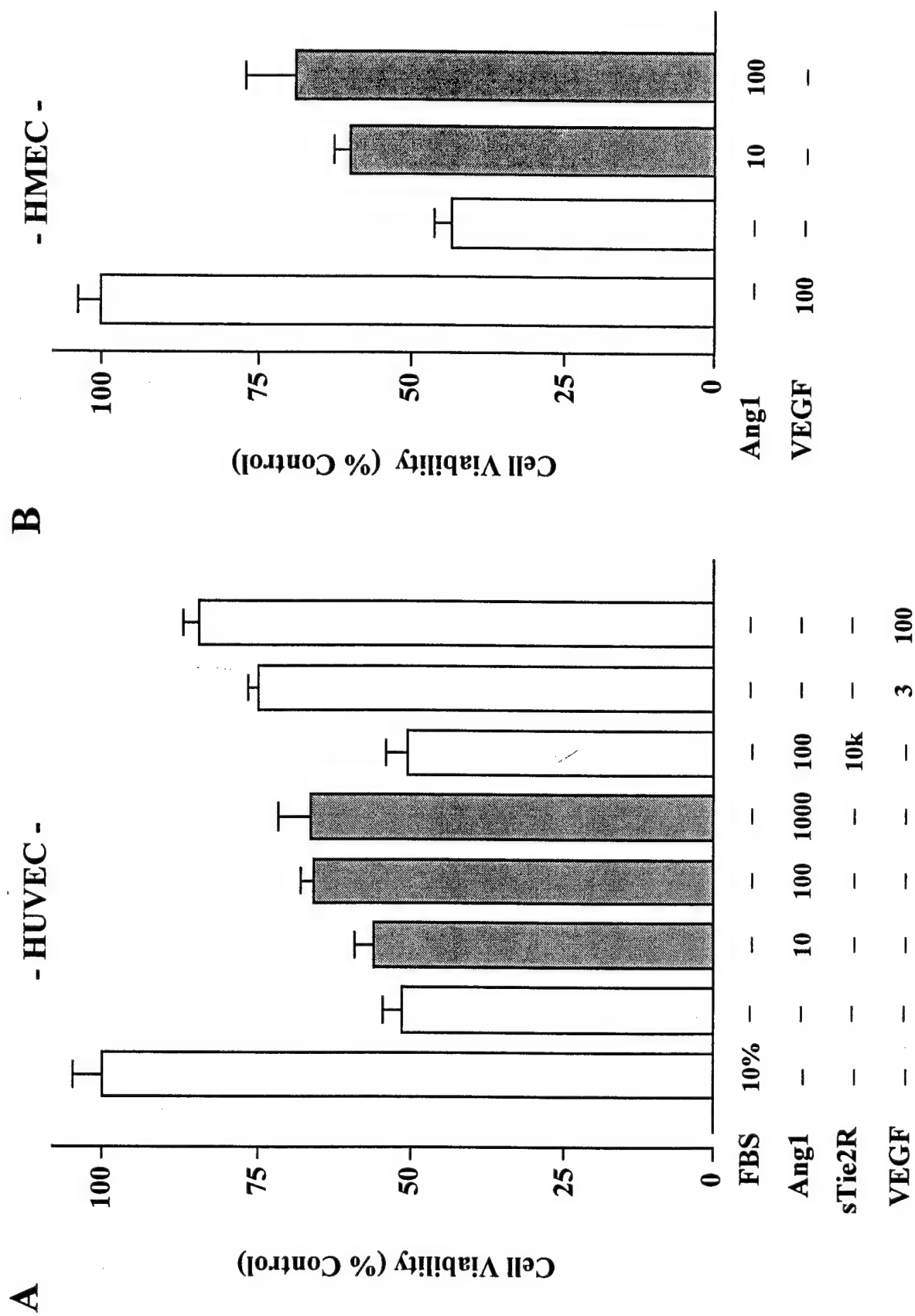
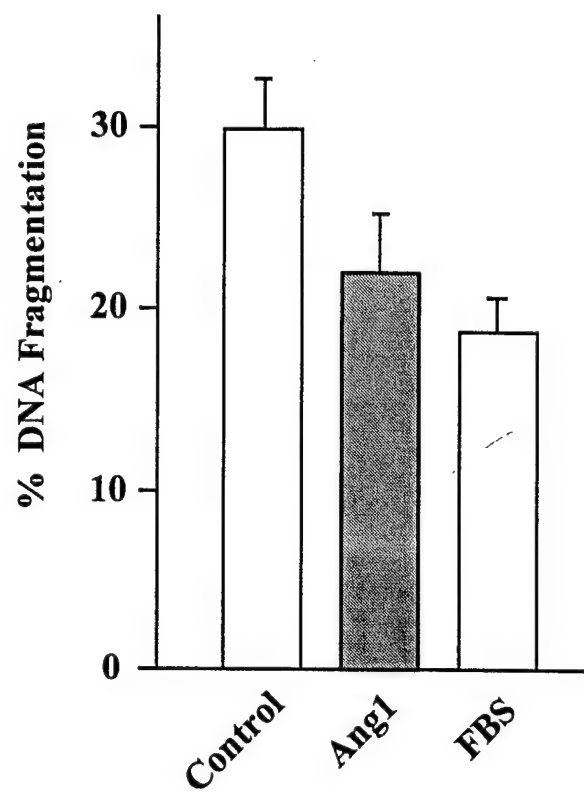


Fig. 2

A



B

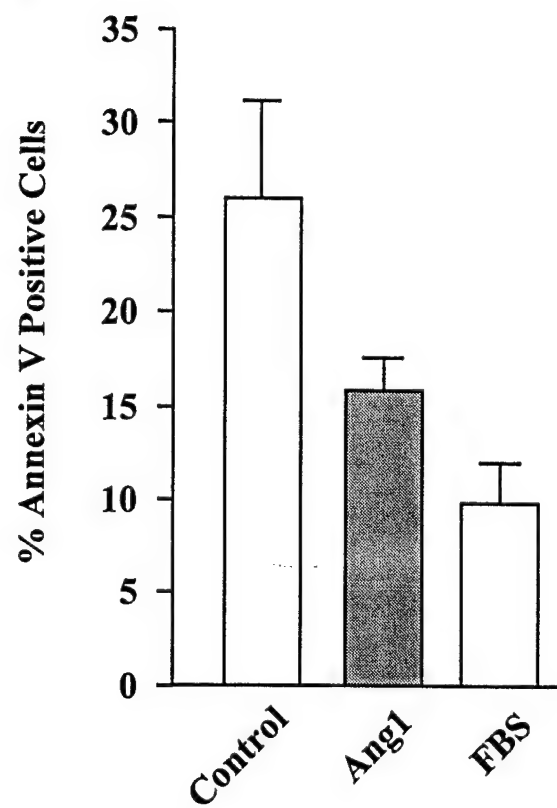


Fig. 3

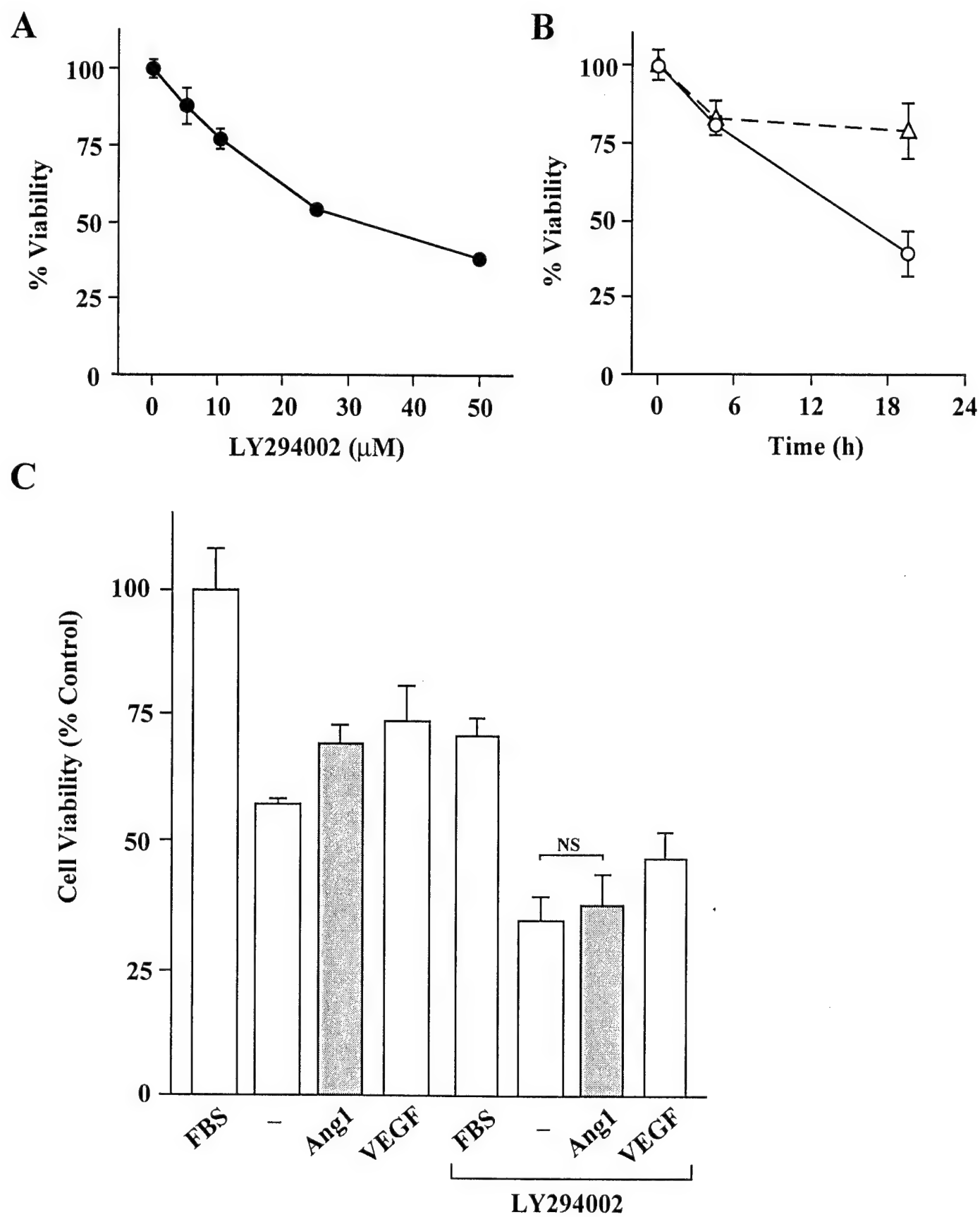


Fig. 3

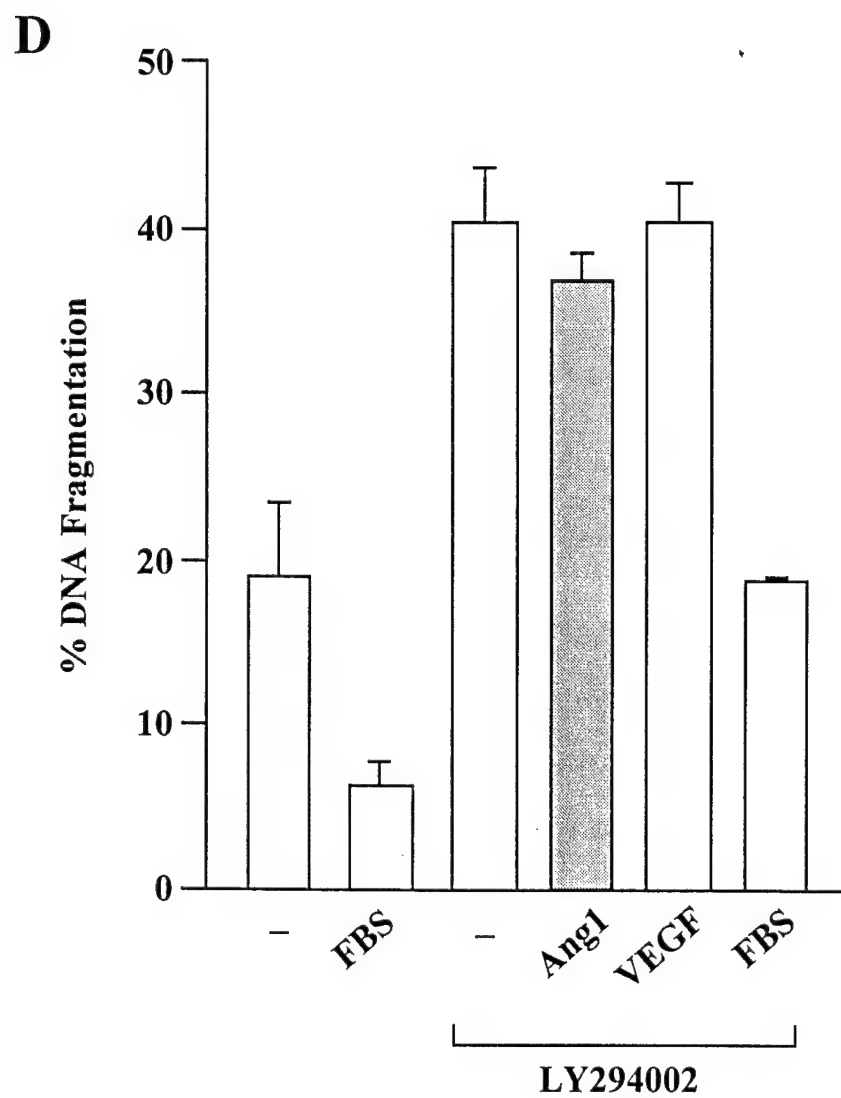


Fig. 4

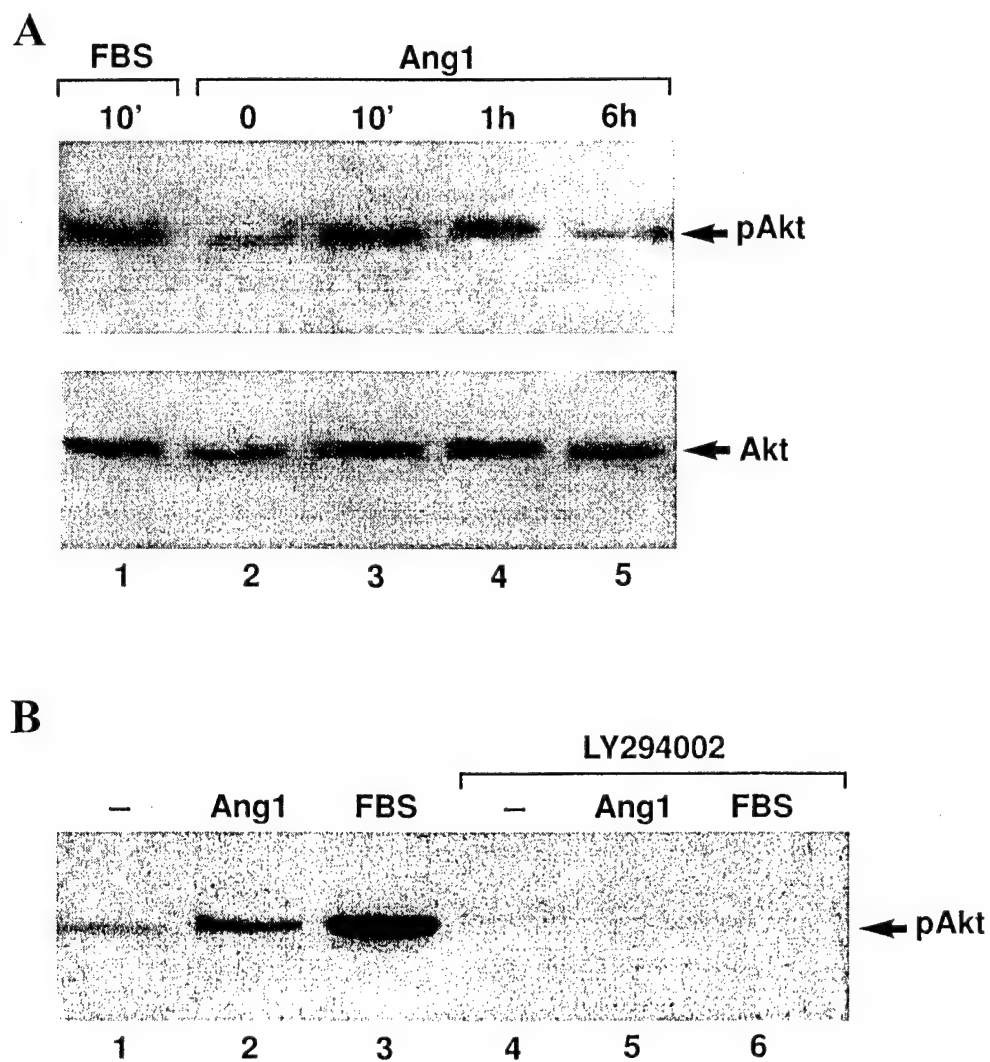


Fig. 5

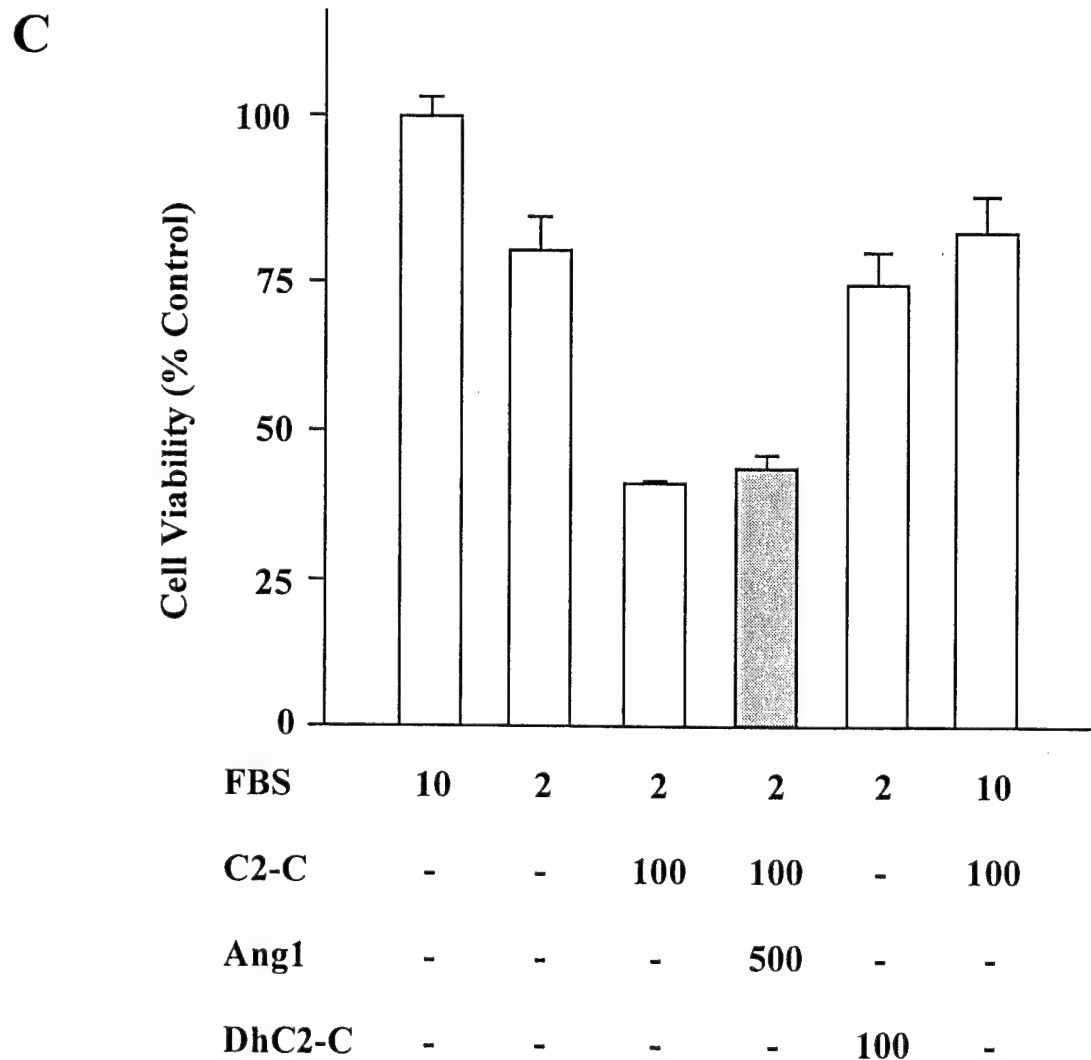
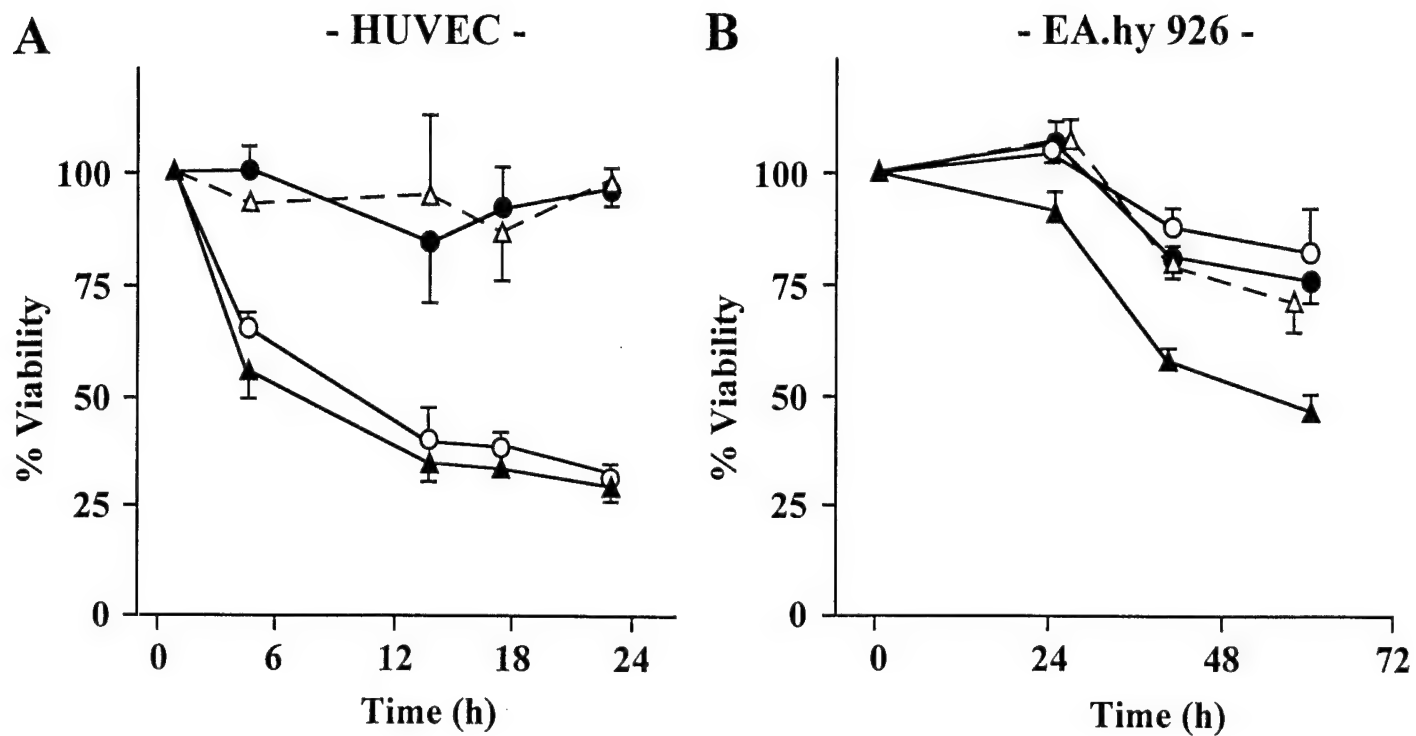


Fig. 5

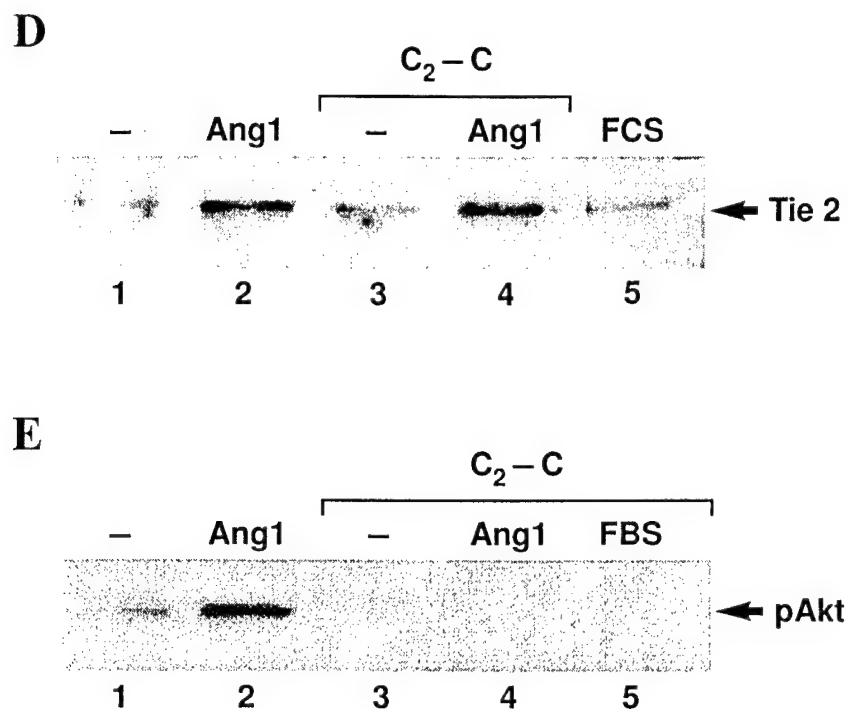


Fig. 6

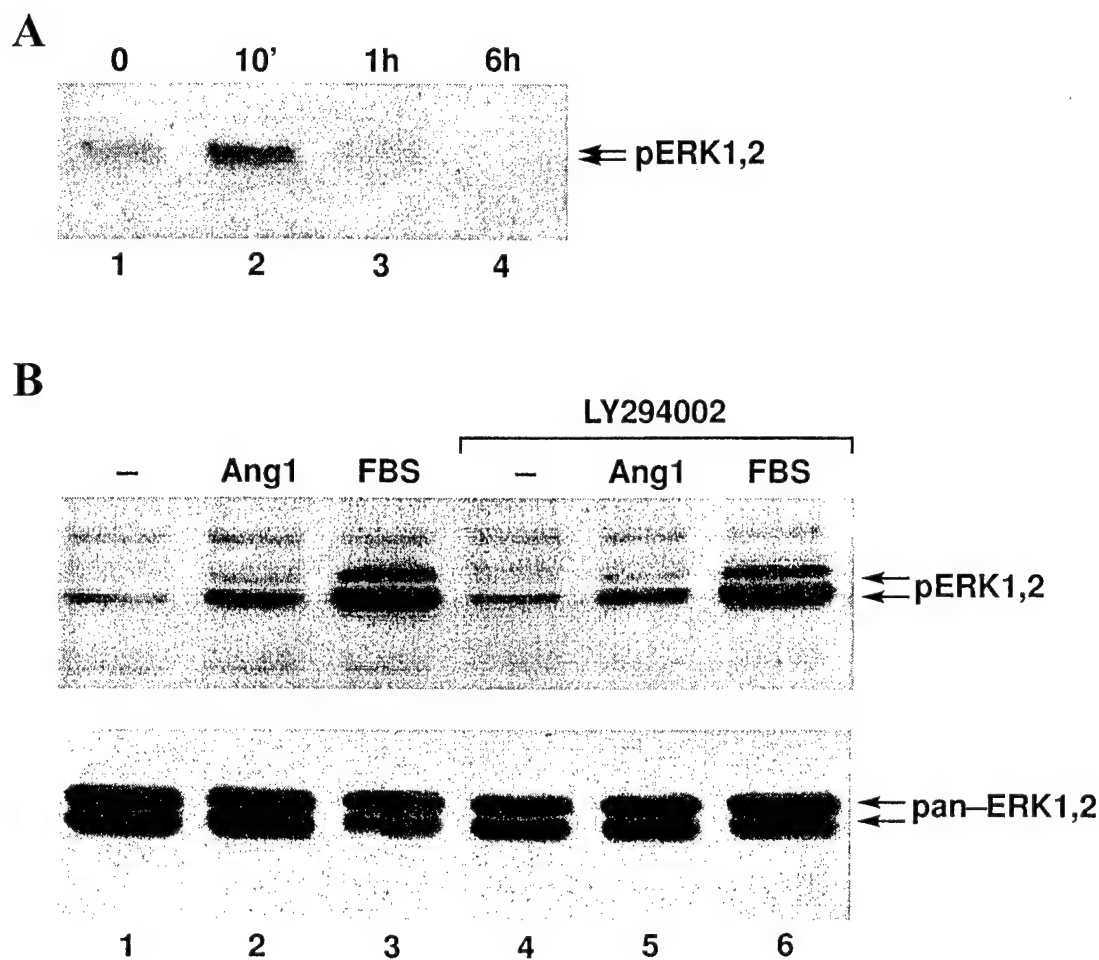


Fig. 6

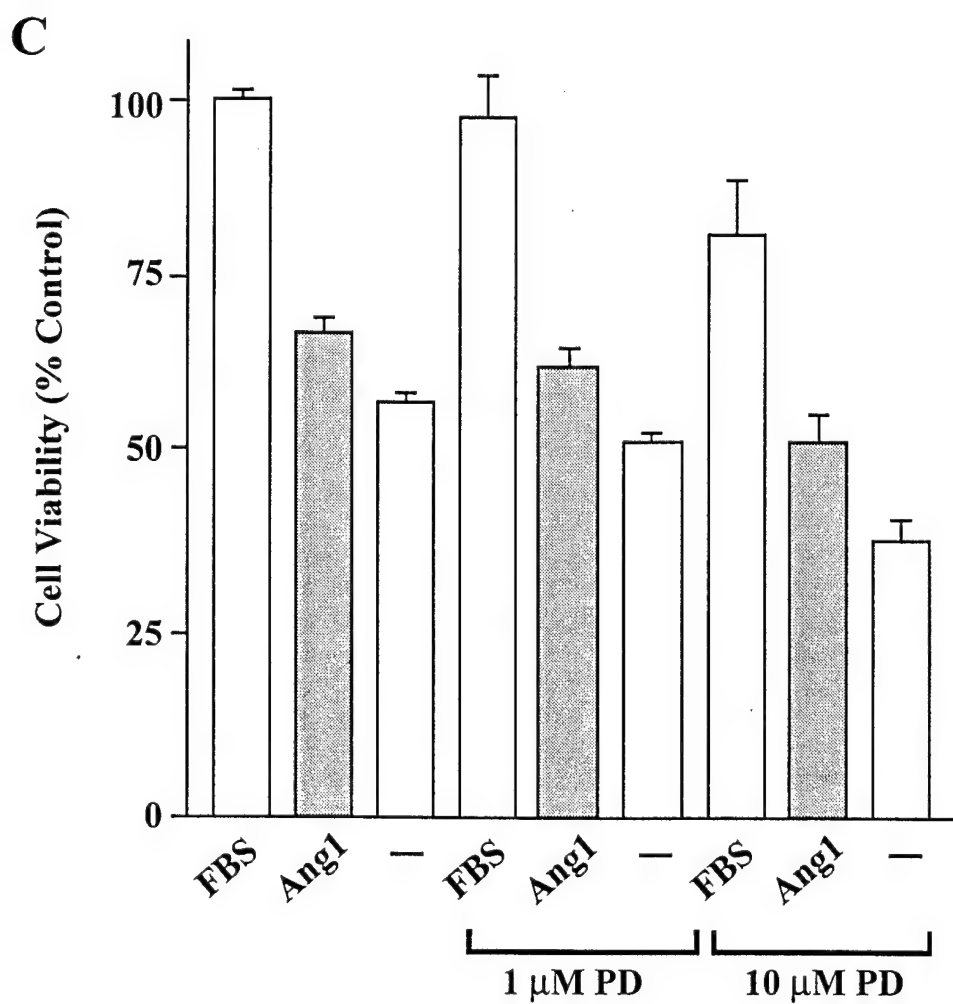
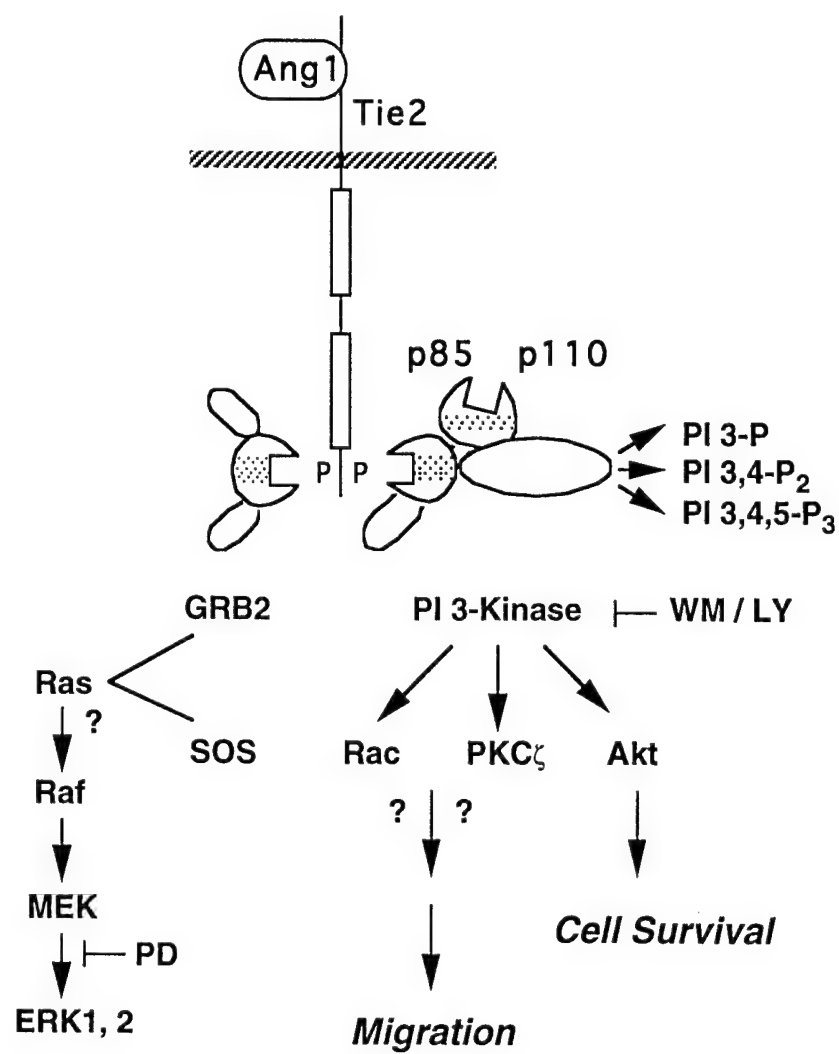


Fig. 7



Expression of Tie1 and Tie2 Proteins during Reendothelialization in Balloon-Injured Rat Carotid Artery

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Key Words

Tie1 · Tie2 · Reendothelialization · Balloon injury · Cell density · Endothelial cell · Platelet

Abstract

The novel endothelial cell tyrosine kinase receptors, Tie1 and Tie2, are essential for vascular development and remodeling in the embryo but little is known regarding the regulation of their expression and their role in the maintenance and repair of the adult vascular system. We examined the expression of Tie1 and Tie2 in normal vessels and during reendothelialization following balloon injury of the adult rat carotid artery. Tie proteins were detected in quiescent endothelial cells of the adult rat carotid artery. Tie1 and Tie2 proteins were also detected in human and rat platelets. A weak expression of Tie1 and Tie2 proteins was detected in young endothelial cells which sparsely repopulated the denuded surface by day 14. Protein levels increased in the confluent layer of endothelial cells by day 28. Based on these observations, we tested whether Tie1 and Tie2 mRNA and protein levels are upregulated by cell density. Tie1 and Tie2 expression significantly increased with higher density in cultured human endothelial cells, and this upregulation required cell-cell interaction. These data suggest that these proteins may play a role in the maintenance and repair of the adult vascular system and that the expression of Tie1 and Tie2 is regulated by cell density.

Introduction

Angiogenesis involves structural remodeling of large and small vessels with development of new vascular networks [1]. It has been suggested that the initiating feature for new blood vessel growth is the activation of endothelial cells, followed by disruption of the basement membrane, and migration of endothelial cells in the direction of a stimulus [2]. Endothelial cell proliferation and survival are regulated by growth factor receptors which have distinct functions. The activation of vascular endothelial growth factor (VEGF) receptors, Flt-1 and KDR, regulates endothelial cell differentiation in the embryo and proliferation in areas of new blood vessel formation throughout adult life [3]. The development of collateral vessels in an ischemic lower limb and during reendothelialization of the balloon-injured carotid artery is augmented by administration of VEGF [4, 5].

Tie1 and Tie2 (tyrosine kinase receptors with immunoglobulin and EGF homology domains) are a novel family of transmembrane receptors [6, 7]. Tie2 ligands, angiopoietin-1 (Ang1) and angiopoietin-2 (Ang2), are also required for angiogenesis in the embryo [8–10] but do not induce endothelial cell proliferation. Transcripts of VEGF receptors, Flt-1 and KDR, and mRNA for Tie2 and Tie1 receptors are detected sequentially at approximately half-day intervals starting at E 7.0 [11], and experiments using knock-out mice confirm that a lack of each receptor results in temporal arrest of embryonic develop-

ment of the vascular system [12–15]. Tie1-deficient mice die few days later than do mice which lack Tie2, Ang1, Flt-1 or KDR. The common features of Tie^{-/-} and Ang1^{-/-} mice are defective vascular branching and capillary development in addition to extensive hemorrhage [14–16]. These observations indicate that Tie1 and Tie2 are essential for the development of the vascular network, whereas VEGF receptors determine early vasculogenesis in the embryo.

The role of Tie and Ang in postnatal life is not well understood. Tie1 and Tie2 expression in adult vessels was reported to decline significantly after birth [6]. Because Ang1 and Ang2 are not mitogenic for cells which express Tie2 receptor, their role in the biology of the adult vascular system has been elusive. Increased expression of Tie2 and its ligands, ~~Tie1 and Tie2~~, occurs at sites of active vascular remodeling during ovulation, wound healing and in metastatic tumors [10, 17–19]. In addition, the gene transfer of Ang1 cDNA to the rabbit ischemic hindlimb accelerates collateral vessel formation [20]. These observations suggest that Tie1, Tie2 and angiopoietins ~~also~~ play a role in remodeling of the vascular system in the postnatal period.

Reendothelialization is an established model to test the expression of proteins associated with vascular repair and endothelial cell remodeling. There is no information on the expression of Tie proteins during reendothelialization. We examined normal and balloon-injured rat carotid arteries for expression of Tie1 and Tie2 and found that their levels are high in mature quiescent endothelial cells and in platelets which adhere to the denuded surface prior to reendothelialization. In addition, an increase in Tie mRNA and protein correlated with cell density during reendothelialization and in cultured endothelial cells. These observations suggest a role for Tie1 and Tie2 in the maintenance of the quiescent state of endothelial cells.

Materials and Methods

Antibodies

Rabbit polyclonal anti-human Tie1 and Tie2 antibodies, blocking peptides and anti-*c-src* antibody were from Santa Cruz Biotechnology (Santa Cruz, Calif., USA). Mouse monoclonal anti-rat CD31 (PECAM-1), anti-smooth-muscle α -actin-alkaline-phosphatase (AP)-conjugated antibody, and biotinylated *Griffonia simplicifolia* lectin I were purchased from SeroTee (Washington, D.C., USA), Sigma Chemicals (St. Louis, Mo., USA), and Vector Laboratories (Burlingame, Calif., USA), respectively.

Cell Culture and Cell Extracts

Human umbilical vein endothelial cells (HUVEC) were isolated from human umbilical cords [21] and grown as previously reported [22]. EA.hy 926, a HUVEC/small cell carcinoma hybrid cell line, was a gift from Dr. C.-J.S. Edgell [23] and was cultured in Dulbecco's modification of Eagle's medium supplemented with 10% fetal bovine serum (FBS). HUVEC and EA.hy-926 cells were grown to confluency, trypsinized and plated at 25, 50, 75, or 100% confluence on gelatin-coated plates (Sigma). After overnight incubation, cells were harvested to extract total RNA or proteins. Jurkat, a human T-cell leukemia cell line, was cultured in RPMI containing 10% FBS. Human platelets were purified from fresh platelet-rich plasma obtained from normal volunteers by differential centrifugation as described [24]. Whole-cell lysates (WCL) were prepared in HEPES buffer, pH 7.40, containing 1% NP-40 [25]. The protein concentration was determined by a Bio-Rad protein assay kit (Bio-Rad Laboratories) using bovine IgG as standard.

To test whether a soluble factor(s) is released from endothelial cells which could regulate Tie expression, HUVEC were cocultured with sparse or confluent HUVEC using gelatin-coated double-chamber plates (Transwell, pore size 0.4 μ ; Fisher, New York, N.Y., USA). Cells at 25% confluency were plated in a lower well, and at 25 or 100% confluency in an upper chamber. Upper chambers were inserted into lower wells and incubated overnight. Total RNA was prepared from HUVEC from lower wells as below.

Western Blotting

50 μ g of WCL protein was boiled in sample buffer and fractionated by 12% SDS-PAGE. Gels were electrophoretically transferred onto nitrocellulose membrane and blocked for 1 h at room temperature [7% non-fat milk and 2% BSA in PBS, 0.1% Tween-20 (PBS-T)]. Membranes were incubated for 3 h at room temperature with Tie1 or Tie2 antibodies in 2% BSA in PBS and washed 4 times with PBS-T. The goat anti-rabbit horseradish peroxidase (HRP)-conjugated antibody was used at 1:3,000 dilution in 2% BSA/PBS-T for 45 min. Following 4 washes in PBS-T, immunoreactive bands were detected using ECL reagent (Pierce, Rockford, Ill., USA) as per manufacturers' instructions.

Balloon Injury of Rat Carotid Artery Model

Male 2- to 3-month-old Sprague-Dawley rats were anesthetized with an intraperitoneal injection of pentobarbital sodium solution (60 mg/kg; Abbott Laboratories, North Chicago, Ill., USA). Deendothelialization injury of the left common carotid artery was performed using the balloon denudation technique established by Reidy et al. [26] and modified by Asahara et al. [5]. The bifurcation of the left common carotid artery was exposed through a midline incision, and blood flow to the site of surgical manipulation was temporarily interrupted. A 2-F Fogarty embolectomy catheter (Baxter Edwards Healthcare, Irvine, Calif., USA) was then introduced through an arteriotomy in the external carotid artery and advanced to the proximal edge of the omohyoid muscle. The balloon was inflated with saline and withdrawn 3 times to produce a distending reendothelialization injury. After removing the balloon catheter, the ligatures applied to the left common and internal carotid arteries were released, and the external carotid artery was permanently ligated. All rats were injected in the tail vein with 0.5% Evans blue dye (Sigma) to identify the denuded area prior to sacrifice. The animals were sacrificed on days 3, 7, 14 and 28. Following perfusion with 100% methanol through the heart, the denuded segment of the left carotid artery

was dissected. Carotid arteries from animals which were not subjected to surgery and the noninjured right carotid artery from experimental animals were used as controls. The experimental protocol for this project was approved by the Institutional Animal Care and Use Committee of St. Elizabeth's Medical Center and complied with the *Guide for the Care and Use of Laboratory Animals* (NIH Publication 86.23, revised 1985).

Immunohistochemistry

Immunostaining was performed on serial sections as previously described [27]. Briefly, endogenous peroxidase was blocked with 3% H₂O₂, followed by incubation in blocking serum (Signet Labs, Dedham, Mass., USA) and primary antibody at the appropriate dilution (Tie1, 1:100; Tie2, 1:400; *c-src*, 1:1,000; CD31, 1:1,000; α -actin, 1:300) overnight at 4°C. Biotinylated anti-rabbit secondary antibody (Signet) for anti-Tie1, Tie2, and *c-src*, or biotinylated anti-mouse rat absorbed secondary antibody (Vector) for anti-CD31 was then applied for 30 min at room temperature followed by streptavidin. Antigens were visualized by incubation with 0.05% (w/v) of 3,3'-diaminobenzidine tetrahydrochloride dehydrate (DAB; Vector). All sections were counterstained with 10% Gill hematoxylin (Fisher). AP-labeled anti- α -actin antibody was visualized by 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium, and slides were counterstained with nuclear fast red (Vector). Biotinylated lectin I was applied to the sections at a dilution of 1:100 followed by streptavidin-HRP, and visualized by DAB.

To confirm the specificity of anti-Tie1 and -Tie2 antibodies, tissue sections were preincubated with a 100-fold excess of specific peptides for 1 h at room temperature before applying to tissue sections. Normal rabbit IgG (Sigma) was used as negative control. Normal mouse IgG (Vector) was substituted for primary antibodies (anti-CD31 and anti- α -actin) and was used as negative control for each experiment.

Double Immunofluorescence

Following deparaffinization and rehydration, the sections were blocked with normal goat serum and incubated with respective antibodies at 4°C overnight. After washing, sections were incubated with rhodamine-labeled anti-rabbit secondary antibody (Pierce) and biotinylated anti-mouse IgG antibody for 30 min. The biotinylated antibodies were detected by extravidin-fluorescein (FITC; Sigma). The sections were mounted in Vectashield mounting medium (Vector) and visualized using a Diaphot microscope (Nikon, Tokyo, Japan).

Transmission Immunoelectron Microscopy

The segments of rat carotid arteries were mounted in Tissue-Tek O.C.T. compound (Miles Laboratories, Naperville, Ill., USA) and frozen in liquid nitrogen. The tissues were cut onto slides, and fixed with 4% paraformaldehyde for 10 min. Following treatment with 0.5 mg/ml sodium borohydride in PBS for 30 min at 4°C and 1% Triton in PBS for 1 min at 4°C, the sections were blocked with normal goat serum, incubated with anti-Tie1 antibody at 4°C overnight, and washed with PBS containing 0.05% Tween 20. Slides were incubated with biotinylated secondary antibody for 30 min and the bound antibodies detected using avidin-10-nm gold reagent (Sigma) incubation for 30 min, fixed with 2.5% glutaraldehyde for 1 h at 4°C and subsequently postfixed with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 1 h at 4°C. Following washing, they were stained en bloc with 2% uranyl acetate, dehydrated with ethanol and embedded in Epon Araldite epoxy resin. The sections were stained

with uranyl acetate and examined with a Philips CM10 electron microscope.

Reverse Transcriptase PCR

Reverse transcriptase PCR (RT-PCR) was performed using total RNA extracted using Ultraspec (Biotex Laboratories, Houston, Tex., USA). 2 μ g of total RNA was reverse-transcribed using oligo dT primers and 200 U of Superscript reverse transcriptase (Life Technologies) at 42°C for 60 min. An equal amount of RT product was used as template DNA for PCR. Amplification was carried out with 0.3 μ M of each sense and antisense primer, 200 μ M dNTP, and KlenTaq-I DNA polymerase (Advantage-GC PCR kit; Clontech Laboratories, Palo Alto, Calif., USA). Primers were designed from the sequences for human Tie1 [7] and Tie2 [28], as follows: Tie1, 5'-CTG AGG AGA CAA GCA CCA TCA TCC-3' and 5'-GGT TCT CTC CGA CCA GCA CAT TCC-3'; Tie2, 5'-AGT AGD CAT ATT CAC CAT CAA CCG-3' and 5'-TGC CAA GCC TCA TAG TGA TTA ACG-3'. Amplification was started with 1 cycle at 94°C for 2 min, 30 cycles at 94°C for 30 s, 64°C for 3 min, and ended with 1 cycle at 64°C for 7 min. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Clontech) was used as control for RNA quality. cDNA from Jurkat cells was used as negative control. PCR products were separated on a 2% agarose gel, and visualized by ethidium bromide staining.

Results

Expression of Tie1 and Tie2 Proteins in Normal Adult Rat Carotid Artery

The rat carotid artery denuded by balloon injury is a reliable model to study endothelial cell proliferation and reendothelialization *in vivo* [5]. Reendothelialization occurs reproducibly in 2–4 weeks. Using antibodies to the C-terminal peptides of Tie1 and Tie2, we performed immunohistochemistry on normal and balloon-injured rat carotid arteries. Tie1 and Tie2 proteins were detected in normal rat carotid artery (fig. 1c, d). CD31 (PECAM-1) antigen (fig. 1a) and lectin I (not shown) were used as endothelial cell markers and α -actin as smooth muscle cell marker (fig. 1b). To determine the specificity of immunostaining by Tie antibodies, tissue sections were incubated with Tie1 or Tie2 antibodies preadsorbed with a 100-fold excess of specific peptides. Anti-Tie1 antibody (fig. 1f) and Tie2 antibody (not shown) binding were completely removed by competing peptides. In addition, no nonspecific staining was observed when normal rabbit IgG (fig. 1e) was used. These results indicate that Tie proteins are present in normal adult quiescent vascular endothelial cells.

To determine whether Tie proteins colocalize with the endothelial cell-specific marker, CD31, we performed double immunofluorescence. Normal rat carotid artery was labeled with anti-CD31/FITC and anti-Tie1/rhodam-

(color illustr.)

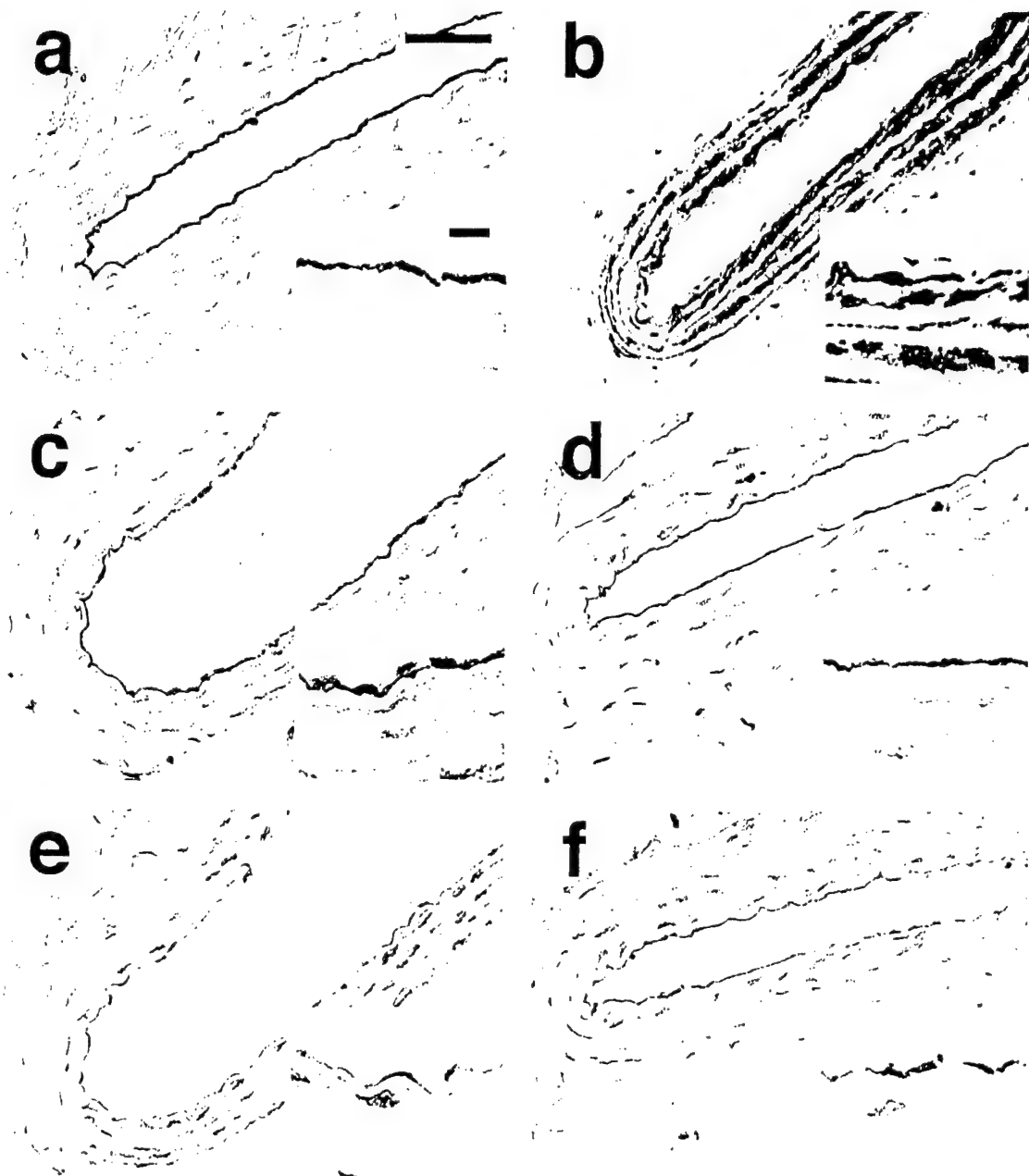
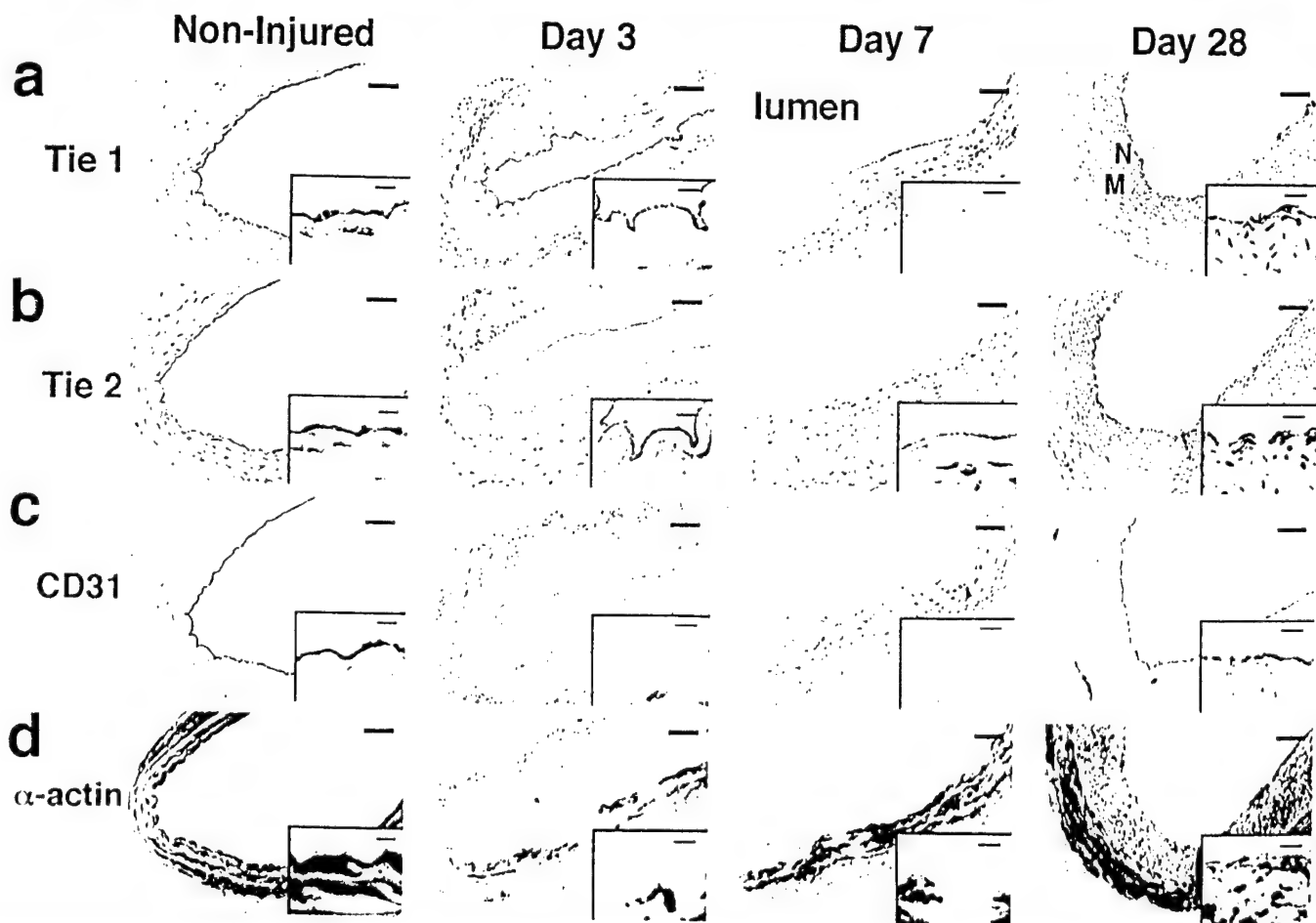
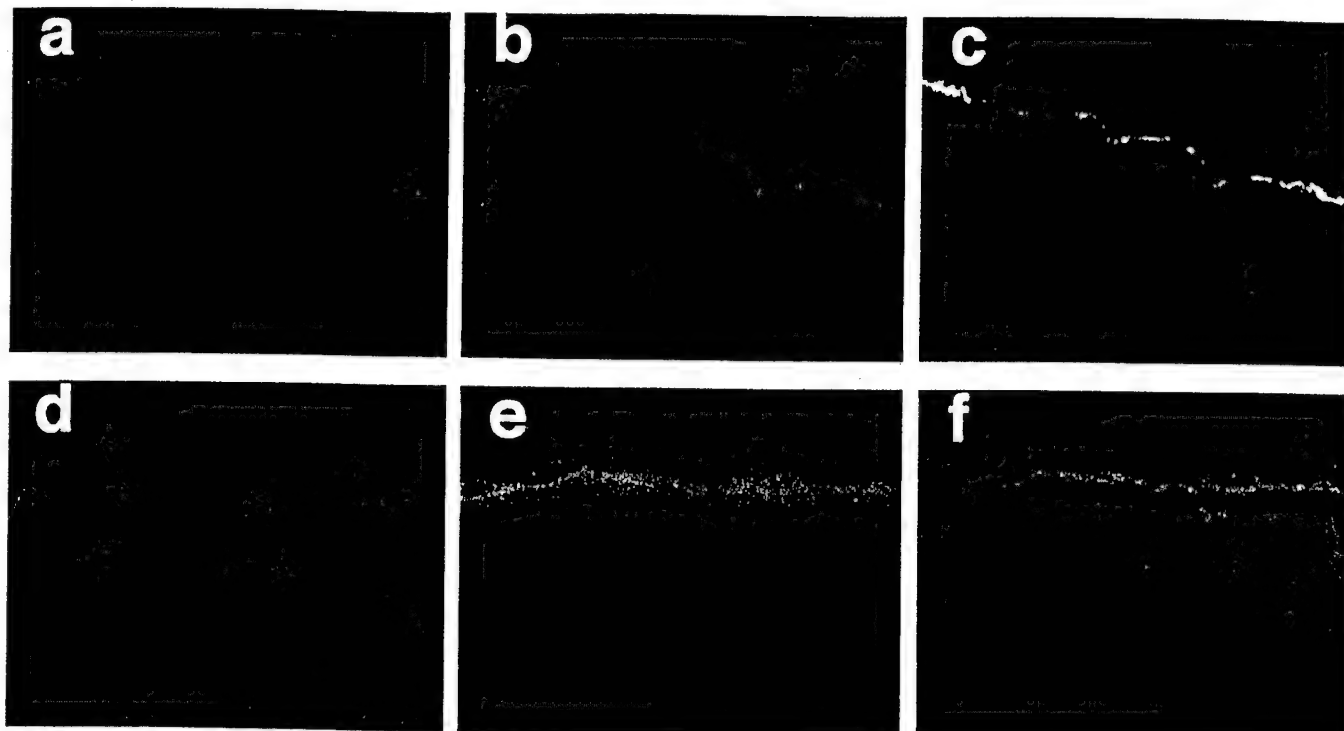


Fig. 1. Immunohistochemical staining of the normal rat carotid artery. The panels show a $\times 100$ magnification (scale bar = 50 μm) while the inserts within squares have a higher magnification ($\times 200$, scale bar = 10 μm). **a** CD31 (PECAM) staining is positive in the endothelial cell layer (brown). A higher magnification (insert) shows endothelial cells in the lumen of the arterial wall. Sections were counterstained with hematoxylin. **b** α -Actin was positive in the media (purple). Sections were counterstained with nuclear fast red (red). **c, d** Tiel (**c**) and Tie2 (**d**) proteins are expressed in endothelial cells of rat carotid artery (brown). The inset shows a higher magnification of the luminal area. Sections were counterstained with hematoxylin. **e** Preadsorption of anti-Tie1 antibody with specific peptides resulted in complete disappearance of immunoreactive stain. **f** Normal rabbit IgG was used as negative control.

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ine-conjugated antibodies. Normal endothelial cells stained with anti-CD31 (fig. 2a, green label) and anti-Tie1 antibody (fig. 2b, red label). Tie1 antibody colocalized with CD31 in endothelial cells which resulted in appearance of orange fluorescence (fig. 2c, merged image). Additional double immunofluorescence was performed using anti- α -actin, a smooth muscle cell marker/FITC. Expression of α -actin was limited to the arterial media (fig. 2d, green label), and Tie1 (fig. 2e, red label) expression was detected only in endothelial cells (fig. 2f, merged image). Similar data were obtained using anti-Tie2 antibody (not shown). These data confirm that Tie1 and Tie2 accumulate in endothelial cells in normal adult rat carotid artery.

Expression of Tie Proteins during Reendothelialization in Balloon-Injured Rat Carotid Artery

The Tie1 and Tie2 protein content did not change in the contralateral rat carotid artery examined on days 3, 7, 14, or 28. In spite of the complete absence of the endothelial layer on the denuded surface 3 days after balloon injury, we observed intense punctate surface labeling with Tie1 and Tie2 antibodies (fig. 3a, b). The specificity of this reaction was confirmed by adsorption with specific peptides. The lack of endothelial cells in these sections was confirmed by the absence of nuclei and staining for other endothelial cell markers: CD31 (fig. 3c), lectin I (not shown), and examination with Evans blue dye. Previous

studies showed that the surface of the balloon-injured artery contains a monolayer of platelets which adhere to the denuded endothelial surface immediately following injury [29]. We found that on day 3 the surface of the denuded arteries also contains platelets. These platelets were identified by immunohistochemistry using anti-*src* antibodies [30] which recognize rat *c-src* protein and a cytospin of isolated human platelets also stained for Tie1 and Tie2 proteins. To confirm that Tie1 and Tie2 proteins are expressed in normal endothelial cells and that the punctate staining of the denuded surface on day 3 after injury corresponds to platelet Tie1 and Tie2 proteins, we performed transmission immune electron microscopy of the normal and balloon-injured rat carotid artery. The expression of Tie in normal endothelial cells was confirmed by immunogold-labeled anti-Tie antibodies (fig. 4a, arrowheads). Sections from 3 days after injury contained Tie1 and Tie2 in platelets adjacent to the denuded lamina propria with no evidence of endothelial cell nuclei on this surface (fig. 4b, and data not shown). These platelets were partially degranulated but contained characteristic light and dense granules (fig. 4b, arrows). No immunogold-labeled Tie antibodies were detected in other cells. Human platelets also express both proteins as confirmed by Western blotting. Therefore, Tie1 and Tie2 are expressed in platelets, which is consistent with previous reports of their expression in cells of hematopoietic origin, specifically in megakaryocytes [31–33].

Immunohistochemistry of rat carotid artery 7 days after injury revealed denuded surfaces lacking endothelial cells, which was confirmed by the staining with Evans blue dye and by the lack of reaction with antibodies to CD31 (fig. 3c). Remaining platelets were stained with Tie1 and Tie2 antibodies (fig. 3a, b).

Reendothelialization of rat carotid artery after 14 days is marked by the reappearance of young endothelial cells. On day 28, these cells expressed CD31 antigen, Tie1 and Tie2 (fig. 3a–c). When immunostaining was performed simultaneously on different tissue sections, lower expression of Tie proteins was evident in the newly deposited endothelial cells which have a large cytoplasm and prominent nuclei as compared to control vessels.

These results indicate that the expression of Tie1 and Tie2 in endothelial cells correlates with reendothelialization following balloon injury and follows the expression of endothelial cell-specific antigen, CD31. In addition, the young endothelial cells which sparsely repopulate the denuded surface have lower expression of Tie1 and Tie2 proteins. These data suggest that the expression of Tie1 and Tie2 is regulated by cell density.

Fig. 2. Double immunofluorescence staining of the normal rat carotid artery. **a, b** Sections are double-labeled with anti-CD31/FITC and anti-Tie1/rhodamine-conjugated antibodies. CD31 is detected in the endothelial cells (**a**, green). Tie1 is visualized in the same layer (**b**, red). CD31 and Tie1 colocalize in endothelial cells of the normal rat carotid artery as shown in the merged image in **c**. The higher background present in rhodamine-labeled slides is subtracted when double filter is used for merged images. **d, e** Sections were double-labeled with anti- α -actin/FITC and anti-Tie1/rhodamine-conjugated antibodies. α -Actin is present in smooth muscle cells in the media (**d**, green). Tie1 was localized in the endothelial cell layer (**e**, red). **f** Merged image. Magnification $\times 500$.

Fig. 3. Immunohistochemical analysis of Tie1 and Tie2, CD31, and α -actin in rat carotid artery following balloon injury. Sections were counterstained with nuclear fast red. All sections are shown at $\times 66$ magnification (scale bar = 50 μ m) and the areas within the squares are at $\times 200$ magnification (scale bar = 10 μ m). N = Neointima; M = media. **a** Tie1 expression in the normal and denuded artery on days 3, 7 and 28, as indicated. **b** Tie2 expression as above. **c** Anti-CD31 immunostaining of endothelial cells. **d** Anti- α -actin immunostaining.

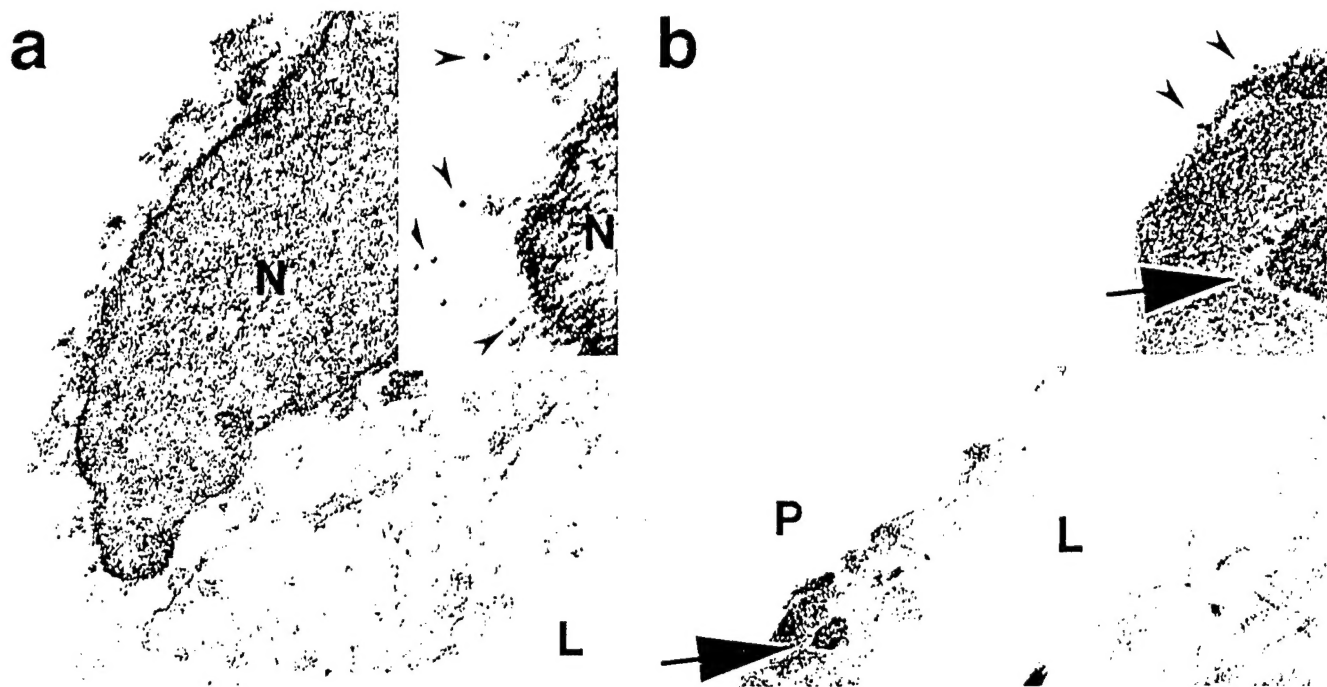


Fig. 4. Transmission immunoelectron microscopic analysis of Tie1 localization in normal and balloon-injured rat carotid artery. **a** Normal rat carotid artery. Immunogold-labeled Tie1 antibodies localize specifically to the endothelial cell. The membrane layer has been disrupted to expose the intracellular epitope. Gold particles are indicated by arrowheads. **b** Immunogold staining of Tie1 in the denuded luminal surface on day 3. Gold particles are visible in platelets (arrowheads) with granules which are partially degranulated (arrow). No nuclei are visualized on the surface in these sections. Magnification $\times 16,900$. The insert shows a higher magnification ($\times 34,600$). N = Nucleus; L = lamina propria; P = platelets.

Tie1 and Tie2 Expression in Cultured Human Endothelial Cells

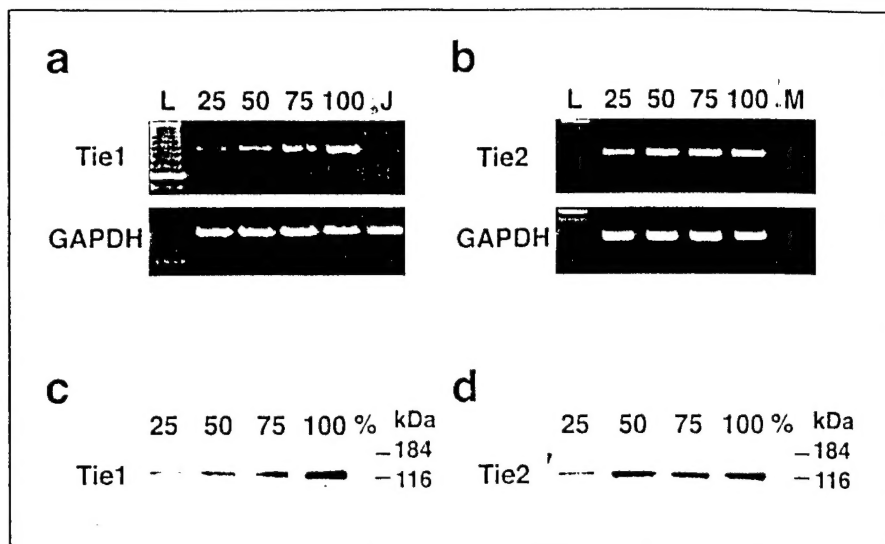
The previous observations suggested accumulation of Tie1 and Tie2 proteins with increased density of endothelial cells. Because of difficulties in obtaining a sufficient number of rat endothelial cells in culture, we used HUVEC and EA.hy 926 cells to test the regulation of Tie1 and Tie2 by cell density. We performed RT-PCR using Tie1- and Tie2-specific primers using RNA isolated from cells plated at different densities. Upregulation of Tie1 and Tie2 mRNA was observed at 16 and 8 h, respectively, in HUVEC (fig. 5a, b) and in EA.hy 926 cells (not shown). Maximal expression of Tie1 was seen at 100% confluency, while Tie2 mRNA was increased at 50% confluency. RNA from Jurkat, a human T-cell leukemia cell line, was used as negative control [7].

To confirm whether changes in mRNA reflect an accumulation of Tie1 and Tie2 proteins in these cells, cell extracts obtained after 16 h of plating at different densi-

ties were resolved by SDS-PAGE, and Tie1 and Tie2 proteins were detected by Western blotting. The accumulation of Tie1 and Tie2 proteins in cells plated at higher density (fig. 5c, d) correlated with mRNA expression. Similar results were observed in EA.hy 926 cells. These data strongly suggest that cell density transcriptionally regulates Tie1 and Tie2 expression.

To test whether endothelial cells at higher density secrete a soluble factor(s) which induces the expression of Tie, HUVEC were cocultured with sparse or confluent cells and Tie1 and Tie2 mRNA was examined by RT-PCR. There was no difference between mRNA levels in cells cocultured with sparse or confluent HUVEC (not shown). These results do not support the hypothesis of a soluble factor responsible for upregulation of Tie, but suggest that direct cell-cell contact is required for upregulation of Tie expression.

Fig. 5. Effect of increasing cell density on Tie1 and Tie2 mRNA and protein expression in HUVEC. Results from 1 of 5 independent experiments for RNA expression and 3 experiments for protein levels. **a** RT-PCR for Tie1 (879 bp) after 16 h of plating cells at 25–100% density as indicated. L = 100-bp DNA ladder marker; J = Jurkat cells. **b** RT-PCR for Tie2 (263 bp) after 8 h of plating cells at 25–100% density as indicated. L = 100-bp DNA ladder marker; M = mock. Equal amounts of total RNA were used for each reaction as indicated by the GAPDH RT-PCR product (983 bp, lower panels). **c** Tie1 Western blot of cell extracts obtained after 16 h of plating. 50 µg of cell extract was loaded on each lane and resolved on 12% SDS-PAGE. **d** Relative levels of Tie2 protein detected by Western blot as above.



Discussion

Although the levels of Tie1 and Tie2 mRNA decline after birth, we found that Tie1 and Tie2 proteins accumulate in endothelial cells of normal rat carotid artery. The expression of Tie proteins in quiescent endothelium was confirmed by transmission immune electron microscopy. The apparent discrepancy between previously reported low mRNA levels [6] and our observations could be due to a low turnover of these proteins in quiescent endothelial cells. A lower transcriptional activity for Tie2 in adult tissues was previously suggested by studies in transgenic mice [34].

The balloon-injured rat carotid artery is a reproducible *in vivo* model to test the expression of proteins associated with vascular repair and endothelial cell remodeling during reendothelialization. Newly deposited endothelial cells appear on the denuded surface approximately 14 days after injury, and reendothelialization is nearly completed by 28 days [5]. We observed that endothelial cells which repopulate the denuded artery after injury contained considerably lower levels of Tie1 and Tie2 proteins as compared to normal resting cells or cells from the contralateral (uninjured) vessel. One reason for the decrease in intensity of immunohistochemical reaction could be because newly deposited endothelial cells have abundant cytoplasm while quiescent endothelium has a thin cytoplasmic layer surrounding the nucleus. This consideration is less plausible for cells analyzed 28 days after injury which are morphologically indistinguishable from normal quiescent endothelium but contained lower levels of Tie1

and Tie2. Therefore, we tested whether Tie1 and Tie2 proteins are upregulated by cell density. A significant increase in Tie1 mRNA and protein levels was detected in human endothelial cells plated overnight at 25–100% confluency. Expression of Tie2 was more sensitive to cell density with maximal increase in mRNA and protein levels in cells at 50% confluency. These results suggest that cell-cell interaction leads to accumulation of Tie gene products. High endothelial cell density is reported to result in secretion of a soluble factor which induces expression of a VEGF receptor, KDR [35]. However, Tie1 and Tie2 mRNA levels were not induced by coculture with confluent cells using the double-chamber method. These results indicate that regulation of Tie expression by cell density requires direct cell-cell contact.

Cell density correlates with the expression of a number of cell surface receptors, including TNFα and integrins [36, 37], while the expression of bFGF mRNA is down-regulated by cell density in some cells [38]. In addition, cell density modulates phosphotyrosine-phosphorylated proteins and specifically activates focal adhesion tyrosine kinases, *src* and FAK [39]. The mechanism of regulation of Tie1 and Tie2 expression by cell density is not known, but our studies suggest that it occurs at the transcriptional level. A recent study found that an increase in Tie1 protein in response to VEGF and hypoxia occurs through a transcription-dependent mechanism [40]. These stimuli may prime the newly deposited endothelial cells for later stages of vessel remodeling. Ang1 also regulates expression of Tie2, as determined by decreased mRNA levels in Ang1^{-/-} mice [8]. Balloon injury of carotid artery dimin-

ishes and disrupts Ang1-producing cells and may result in a decreased local availability of Ang1. The levels of Ang1 during reendothelialization are not known and will require further studies.

Studies using transgenic mice suggest that Tie gene products are required for the integrity of the microvasculature. In chimeric animals, cells which lack Tie1 are unable to contribute to the vascular network of an organ which develops late in the embryo, such as the kidney [15]. Therapeutic administration of VEGF or bFGF accelerates reendothelialization of balloon-injured rat carotid artery [5, 41]. ~~Although Ang1 is not mitogenic for cells which express Tie2 receptors,~~ administration of Ang1 cDNA also improves vascularization of the ischemic hindlimb in rabbits [20]. Our study shows that expression of Tie1 and Tie2 marks the extent of reendothelialization of the balloon-denuded artery which suggests a role for these receptors in reendothelialization and survival of endothelial cells. Our data are also consistent with a previous report [42] which proposed a role for Tie2 in the maintenance of normal quiescent adult vasculature in the postnatal period.

Initial studies using ¹in situ hybridization localized Tie1 and Tie2 exclusively to vascular endothelial cells [14, 18]. Subsequent studies showed that Tie1 is also expressed in early hematopoietic precursors. Expression of Tie1 and Tie2 in megakaryoblastic and erythroleukemia cell lines has recently been reported [31, 33]. These findings are not surprising given that hematopoietic and endothelial cells are derived from a common precursor in the embryo, the hemangioblast, and share many other markers, including CD34 and VEGF receptors, KDR and

Flt-1. We found that the balloon-injured rat carotid artery contains partially degranulated platelets on the denuded surface 3 days after injury, and rat and human platelets were positive for expression of Tie1 and Tie2. Cell surface expression of Tie1, as detected by FACS analysis was previously reported [33]. *in platelets*

The formation of large platelet thrombi is described immediately following metallic filament injury of rat carotid artery while a monolayer of platelets which adhere to the denuded surface is described after balloon-induced injury [41]. A greater concentration of platelets at the site of injury has been proposed to provide mitogens which subsequently induce vascular smooth muscle cell proliferation and delay endothelial regeneration [41]. Expression of Tie1 and Tie2 in hematopoietic precursors is probably redundant because mice which lack Tie1 or Tie2 genes do not have a detectable impairment of the hematopoietic system. However, the extensive hemorrhage observed in the late stage of embryonal development in mice which lack Tie1 or Tie2 may be caused by a qualitative platelet defect in addition to the defective microvascular development described in these animals. Whether platelet function is modulated by engagement of Tie receptors is not known and will require further study.

Acknowledgments

The authors thank Marianne Kearney and Marilyn Keeler for ⁷technical support and Kevin Krasinski for surgical assistance. This work was supported in part by the American Heart Association Fellowship No. 13-429-967 (K.F.), DAMD 17-97-17265 (L.V.) and RO1 NIH-967031 (J.I.).

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